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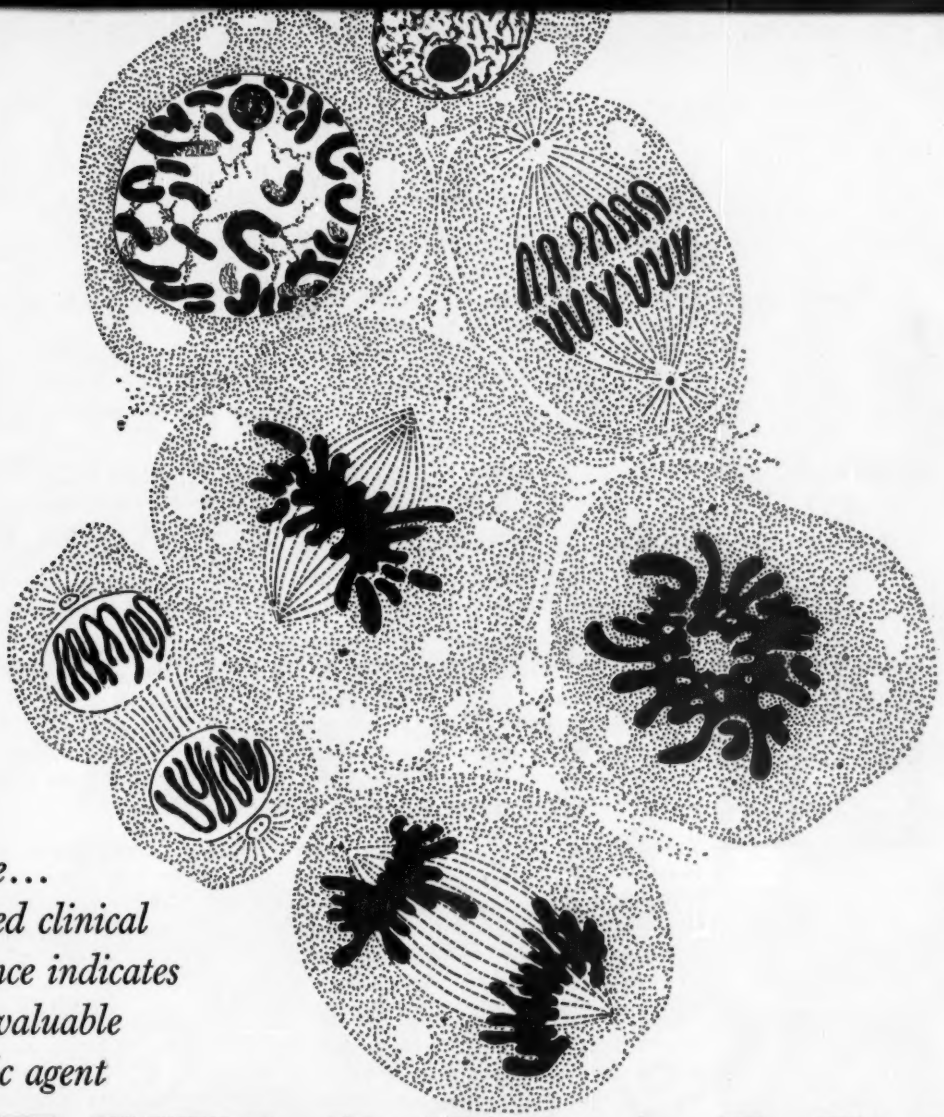

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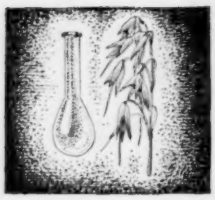

IN JULY—(1922)—the Journal of Biochemistry (Tokyo) reports the isolation and analysis of the prolamin of *Hato-mugi* (Coix lacryma L.). Hato-mugi is an Asiatic species of grass with large seeds called "Job's Tears," which are often strung as beads to pacify teething infants. Results indicate that it contains glutamic acid, leucine, tyrosine and the basic amino acids, arginine, histidine, and lysine, and resembles the prolamin of oats.[1]

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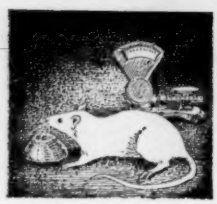

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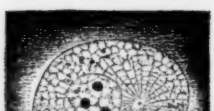

IN JULY—(1959)—discussing the synthesis of organic compounds on the primitive earth, Stanley Miller and Harold Urey state: "The major problems remaining for an understanding of the origin of life are (i) the synthesis of peptides, (ii) the synthesis of purines and pyrimidines, (iii) a mechanism by which 'high-energy' phosphate or other types of bonds could be synthesized continuously, (iv) the synthesis of nucleotides and polynucleotides, (v) the synthesis of polypeptides with catalytic activity (enzymes), and (vi) the development of polynucleotides and the associated enzymes which are capable of self-duplication."[3]

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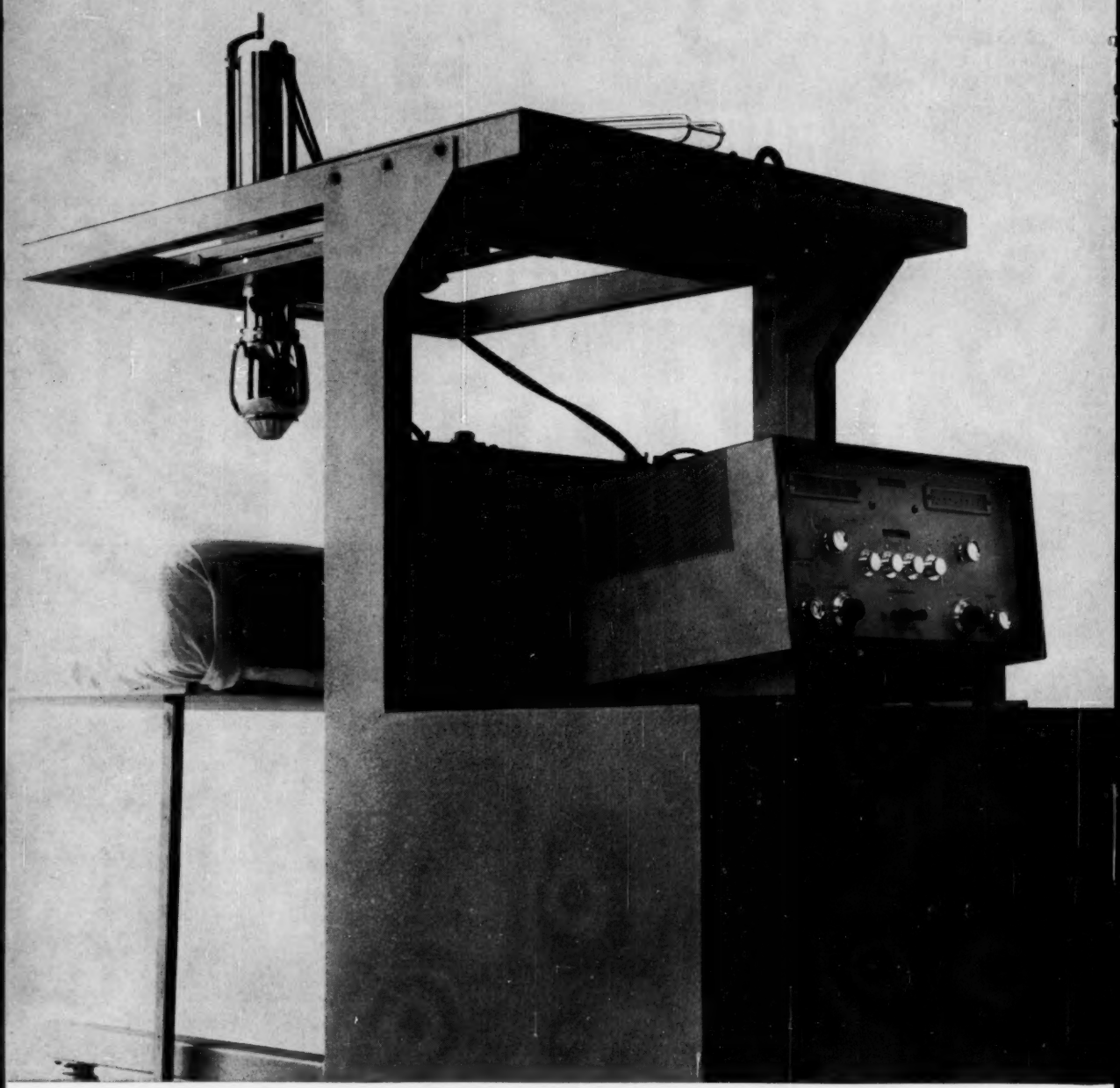

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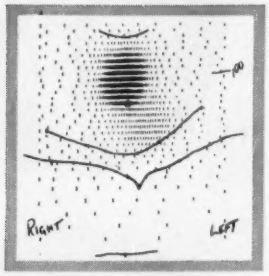
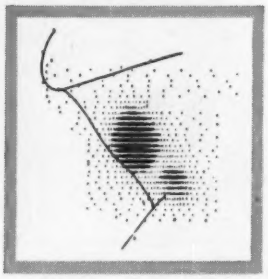
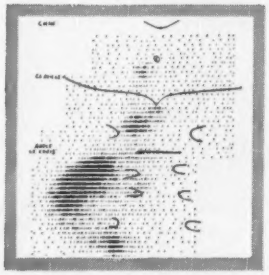


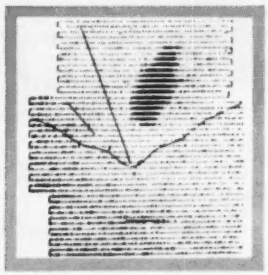
Thyroidectomy in 1928. Thyrotoxic symptoms in 1956. Scan shows recurrence in pyramidal region.



Same patient as top scan. Left lateral view. Gamma-ray Spectrometer used for background reduction.



Intrathoracic recurrence 20 years after surgery. Gamma-ray Spectrometer used with Scaler.



Scan showing residual thyroid area following surgery and node dissection. Spectrometer not used.

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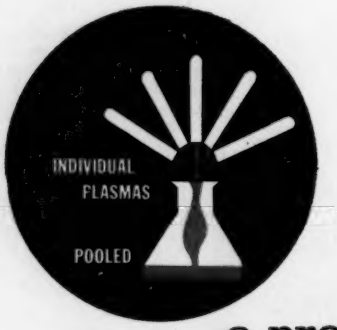

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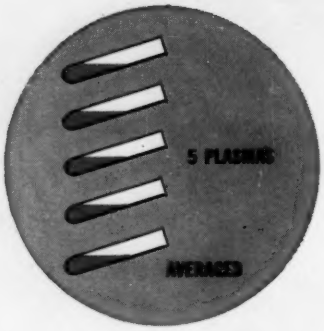

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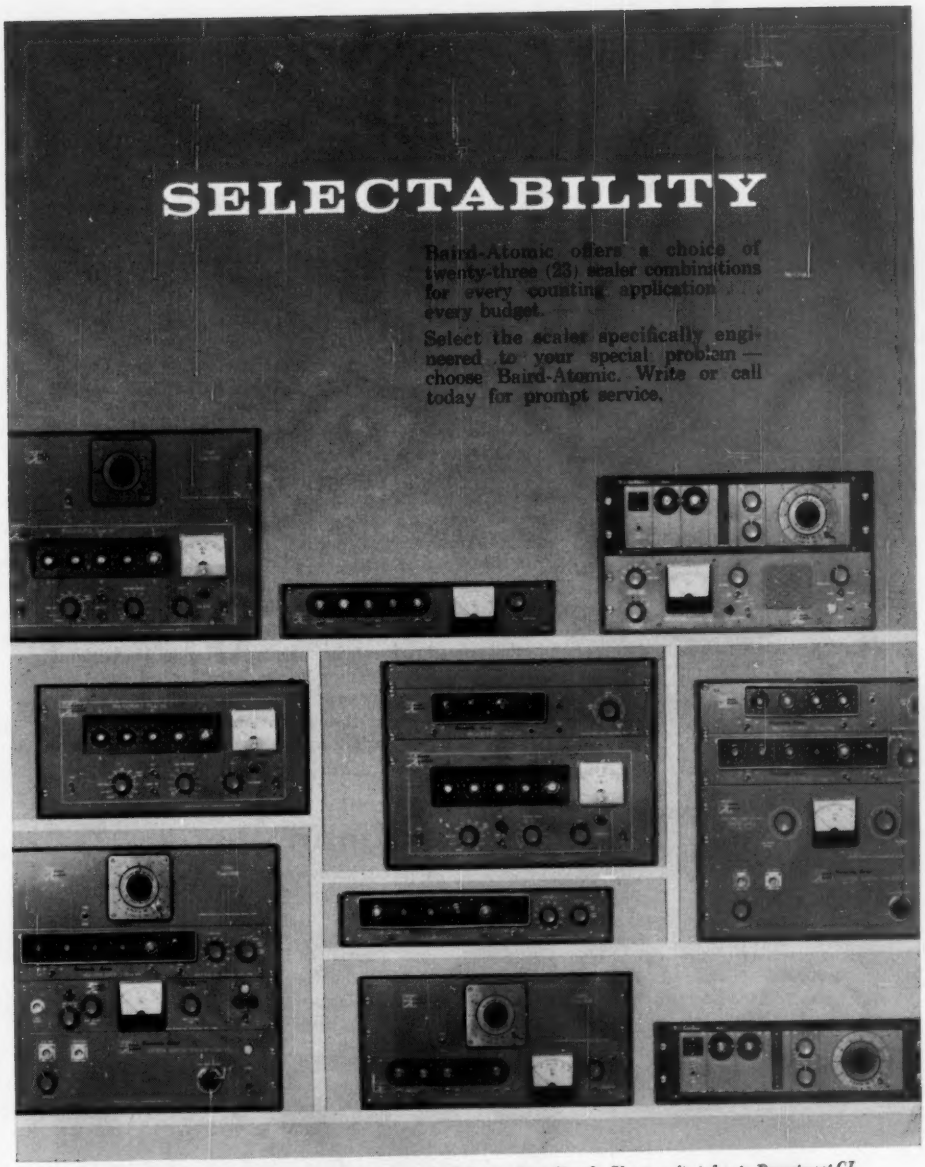
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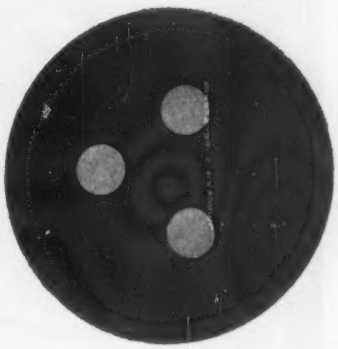

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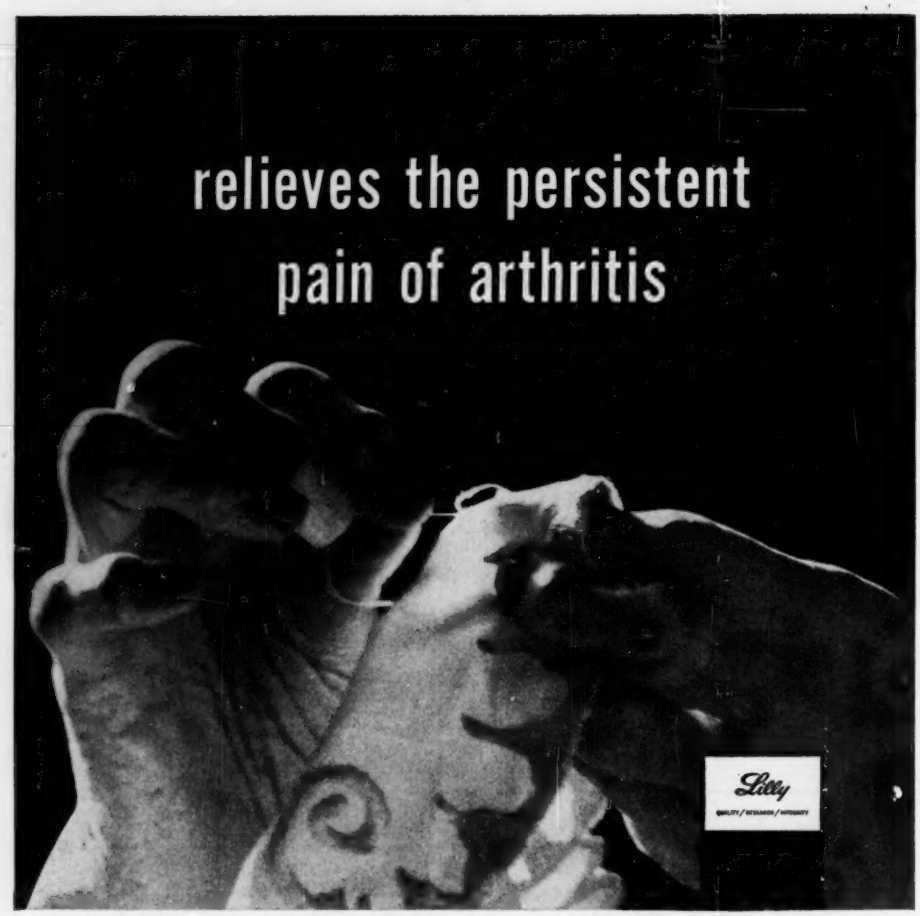

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STUDIES ON STARLING'S LAW OF THE HEART. I. THE CIRCULATORY RESPONSE TO ACUTE HYPERVOLEMIA AND ITS MODIFICATION BY GANGLIONIC BLOCKADE

By ROBERT L. FRYE and EUGENE BRAUNWALD with the technical assistance of ESTELLE R. COHEN

(From the Section of Cardiology, Clinic of Surgery, National Heart Institute, Bethesda, Md.)

(Submitted for publication January 21, 1960; accepted March 4, 1960)

The application of Starling's law of the heart to the circulation of man has been the subject of intensive interest and investigation. Infusions of intravenous fluids have been employed in attempts to simulate in man the increase in venous return to the Starling heart-lung preparation. A variety of fluids has been utilized in such studies in man, including saline (1–4), dextran (4–6), glucose solutions (4), and human serum albumin (2, 7). In general, the results have been conflicting, with a few investigators noting an increase both in filling pressure and cardiac output (1, 5), while in the majority of studies no consistent relationship between these parameters was noted (2–4, 6, 7). Indeed, in most human subjects the augmentation of the total blood volume has failed to be associated with a rise in cardiac output, even in the face of a decline in hematocrit (2–4, 6, 7). The possibility was considered that the presence of an actively functioning autonomic nervous system in the intact state and its absence in the heart-lung preparation accounts for the difference in the cardiac response to infusion in man and increased venous return to the heart-lung preparation. The present study was therefore designed to determine whether the circulatory response to acutely induced hypervolemia is modified by reducing the activity of the autonomic nervous system by means of ganglionic blockade.

METHODS

Seven male subjects with normal cardiovascular systems were studied. They ranged in age from 17 to 39 years and averaged 27 years. The subjects were studied following complete recovery from an acute illness unrelated to the cardiovascular system. Each individual was subjected to three phlebotomies over a period of approximately 10 days and the 1,500 ml of blood so obtained was stored in solutions of sodium citrate, citric acid and dextrose. Approximately two days after the third phlebotomy the first study was carried out. Cardiac output, heart rate, and arterial pressure were recorded, generally in duplicate, immediately before the blood which had been removed was returned. The 1,500 ml transfusion was given at a rate of 19.3 ml per minute utilizing a Bowman infusion pump. Measurements were again carried out immediately following completion of the transfusion and were usually repeated 15 minutes later. Following another series of phlebotomies over a period of approximately 5 days, the procedure was repeated in an identical manner except that the circulatory effects of transfusion were now studied after partial ganglionic blockade had first been induced and while it was maintained through the entire period of observation. In two subjects, H. S. and R. W., the experimental sequence was reversed and the circulatory responses to transfusion were first studied in the presence of ganglionic blockade and the effects of transfusion without blockade were determined at the time of the second study. All observations were carried out with the subjects in the recumbent position, in the postabsorptive state after receiving 100 mg pentobarbital intramuscularly approximately one hour before study.

The ganglionic blockade was induced by a constant, continuous intravenous infusion of Arfonad, a thiophanium derivative (*d*-3,4 [1′,3′-dibenzyl-2′-keto-imidazolido]-1,2-trimethylene thiophanium *d*-camphorsulfonate), a drug which also has some direct vasodilatory action (8, 9). Arfonad was administered by means of an infusion pump at a rate which ranged from 1.0 to 10.4 mg per minute with an average rate of 4.0 mg per minute. The specific infusion rate chosen for each subject was that which lowered systolic arterial pressure by approximately 40 mm Hg and this rate was maintained constant for the entire study. The actual decline of systolic arterial pressure ranged from 32 to 55 mm Hg with an average of 43 mm Hg. Arterial pressure was measured through an indwelling needle with a Statham pressure transducer and recorded, together with the electrocardiogram and the indicator dilution curves, on a multichannel photographic recorder.

Cardiac output was determined by the indicator dilution technic and calculated in the manner described by Hamilton, Moore, Kinsman and Spurling* (10). Evans blue dye was injected from a tared syringe through a 75 cm polyethylene catheter (PE no. 50, 0.58 mm ID) introduced percutaneously through an antecubital vein

TABLE I

Hematocrits of subjects studied

Subj.	Hct. prior to phlebotomy	Initial study		Second study	
		Hct. at beginning	Hct. at completion	Hct. at beginning	Hct. at completion
C. B.	47.5	39.9	38.0	38.5	39.5
H. L.		37.0	35.5	35.0	39.0
M. J.	46.0	34.0	38.0	32.0	36.0
M. Z.	44.0	34.0	34.5	33.5	35.0
H. S.*	35.0	32.0	35.0	32.0	37.0
E. B.	45.5	37.0	38.0	37.0	38.0
R. W.*	43.0	31.0	35.2	29.0	34.0
Avg.	43.5	35.0	36.3	33.9	36.9

* Subjects in whom transfusion was given first in the presence of ganglionic blockade.

into the superior vena cava or right atrium. Arterial blood was sampled through an indwelling arterial needle and pulled through a cuvet densitometer (11, 12) by a motor driven syringe at a rate of 20 ml per minute. The response of the densitometer to a sudden change in density is 95 per cent in one second and over 99 per cent in two seconds (13). The volume of the connecting tube from the arterial needle to the densitometer was 0.41 ml; the time required for the blood to traverse this tubing (1.23 seconds) was subtracted from the observed mean circulation time in calculating the actual mean circulation time. The validity of the technics employed in the measurement of the cardiac output and central blood volume had first been demonstrated by a comparison with direct measurements in a circulatory model (14) and by flowmeter measurements in the open-chest dog over a wide range of flow rates (15).

In the present study 23 of the 28 measurements of cardiac output were carried out in duplicate, 10 to 15 minutes apart. The standard error of the 23 duplicate determinations of cardiac output was 493 ml per minute. Central blood volume, that is, the volume of blood between the site of injection in the vena cava or right atrium and the site of sampling in the systemic artery, as well as all temporally equidistant points, was calculated from the formula: $CBV = \frac{MCT \times C.O.}{60}$ where CBV = central blood volume in liters, MCT = mean circulation time in seconds and C.O. = cardiac output in liters per minute. The standard error of 23 duplicate determinations of the central blood volume was 115 ml. The average of the two cardiac output and central blood volume determinations is presented in Table II in the 23 of the 28 determinations in which they were carried out.

TABLE II

*Hemodynamic data obtained before and after transfusion in the absence and presence of ganglionic blockage**

Subj. BSA	Condition	C. O.		MAP		SV		CBV		MWLV		SWLV	
		Avg. before	Avg. after	Avg. before	Avg. after	Avg. before	Avg. after	Avg. before	Avg. after	Avg. before	Avg. after	Avg. before	Avg. after
C. B.	C	8.97†	7.80†	78†	83†	100†	78†			10.03†	9.30†	111.4†	93.0†
1.86	A	7.03	9.67	75	84	69	108			7.55	11.63	73.9	129.2
H. L.	C	6.19	6.40	109	120	97	92	1.67	1.49	9.69	11.00	152.0	157.1
1.86	A	6.47	8.11†	83	116†	83	101†	1.99	2.20†	7.72	13.09†	97.7	163.6†
M. J.	C	5.41	8.47	78	103	128	146	1.44	1.57	5.98	12.51	139.2	214.6
1.90	A	5.57	8.83	72	106	62	116	1.07	1.48	5.74	13.51	65.6	176.5
M. Z.	C	5.52	4.93	115	123	81	72	1.38	1.33	8.92	8.67	129.3	124.8
1.92	A	4.25	6.52	73	105	56	80	1.05	1.38	4.49	9.88	58.6	122.0
H. S.‡	C	6.22	6.82	83	122	82	73	1.20	1.07	7.42	11.90	97.6	130.6
1.68	A	4.78	6.89	62	93	49	72	0.62	0.90	4.24	9.12	42.9	95.6
E. B.	C	6.03	7.27	82	90	78	86	1.43	1.55	7.06	9.39	91.2	110.7
1.86	A	5.03	6.85	55	88	53	76	1.18	1.35	3.87	8.63	41.4	96.5
R. W.‡	C	6.31	6.46	69	102	103	106	1.50	1.56	6.22	9.42	101.9	154.9
	A	6.25	9.33	56	93	76	127	1.17	1.86	4.98	12.40	60.1	169.7
	C	6.38	6.88	88	106	96	93	1.44	1.43	7.90	10.31	117.5	140.8
Avg.	A	5.63	8.03	68	98	64	97	1.18	1.53	5.51	11.18	62.9	136.2

* BSA = body surface area in m^2; C = control study; A = transfusion in presence of Arfonad infusion; C. O. = cardiac output in L/min; MAP = mean arterial pressure in mm Hg; SV = stroke volume in ml; CBV = central blood volume in liters; MWLV = minute work of left ventricle in kg-m/min; SWLV = stroke work of left ventricle in gram-meters.

† Single determinations.

‡ Subjects in whom transfusion was given first in presence of ganglionic blockade.

The following formula was utilized to calculate the work of the left ventricle:

$$\text{MWLV} = \frac{\text{MAP} \times 1.055 \times \text{C.O.}}{1{,}000}$$

where MWLV = minute work left ventricle in kilogrammeters per minute, MAP = mean arterial pressure in centimeters of H_2O, 1.055 = specific gravity of blood and C.O. = cardiac output in liters per minute.

Again, the average of the two cardiac output determinations was utilized, when available, in these calculations. The statistical analysis is based upon a comparison of the standard deviations of the mean differences in response to transfusion carried out in the absence and in the presence of ganglionic blockade.

RESULTS

Mild anemia was induced by the phlebotomies. The hematocrit values obtained prior to phlebotomy, before, and after both transfusions are presented in Table I. The results of the pertinent hemodynamic measurements are presented in Table II.

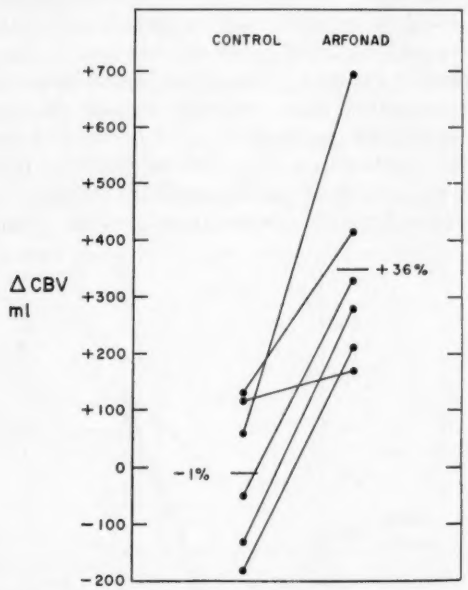

FIG. 1. EFFECT OF ACUTE HYPERVOLEMIA ON CENTRAL BLOOD VOLUME. The solid circles on the left represent the changes in central blood volume produced by the transfusion in the absence of ganglionic blockade. On the right are represented the changes in central blood volume produced by transfusion during ganglionic blockade by Arfonad. The lines between the circles connect observations made on the same subject. The horizontal bar represents the mean change for all subjects and the mean percentage change resulting from transfusion is recorded beside the horizontal bar.

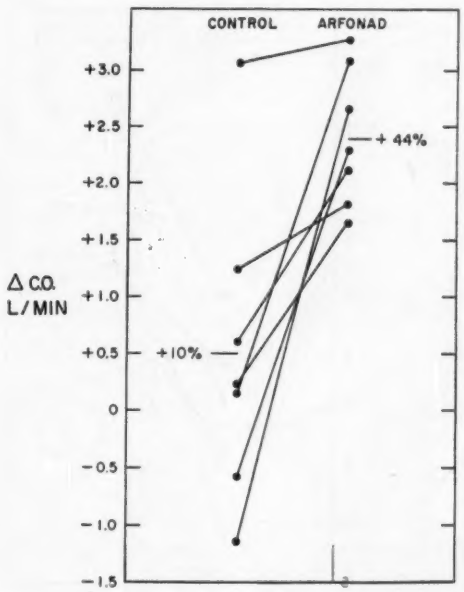

FIG. 2. EFFECT OF ACUTE HYPERVOLEMIA ON CARDIAC OUTPUT.

Figure 1 demonstrates the effect of the transfusion on the central blood volume during the control study and in the presence of partial ganglionic blockade. During the control study the change in central blood volume ranged from − 180 to + 130 ml and averaged − 8 ml, representing an average decline of 1 per cent. In the presence of ganglionic blockade the change in central blood volume ranged from + 170 to + 690 ml and averaged + 350 ml, representing a mean increase of 36 per cent. In each subject the central blood volume rose to a greater extent when transfusion was carried out in the presence of partial ganglionic blockade than in its absence. This difference in response to blood transfusion was statistically significant ($p < 0.01$).

The effect of the transfusion on the cardiac output during the control study and in the presence of Arfonad infusion is represented in Figure 2. During the control study the change in the cardiac output ranged from − 1.17 to + 3.06 L per minute and averaged + 0.50 L per minute, representing a mean increase of 10 per cent. In the presence of partial ganglionic blockade the change in cardiac

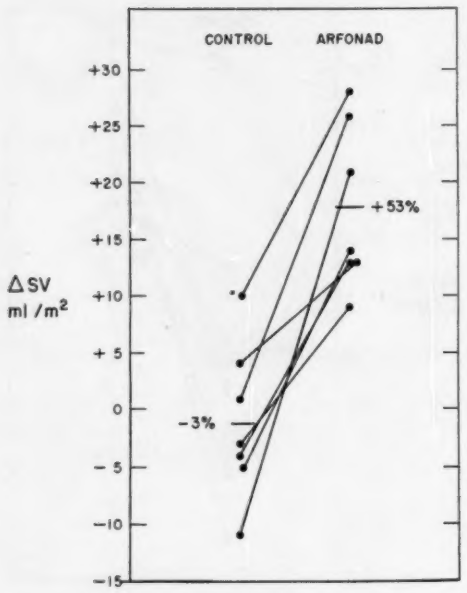

FIG. 3. EFFECT OF ACUTE HYPERVOLEMIA ON STROKE VOLUME.

output ranged from + 1.64 to + 3.26 L per minute and averaged + 2.40 L per minute, representing a mean increase in cardiac output of 44 per cent. In each subject the cardiac output rose to a greater extent when transfusion was carried out in the presence of partial ganglionic blockade than in its absence. This difference in response to transfusion was statistically significant ($p < 0.01$).

Figure 3 illustrates the change in stroke volume index induced by the transfusion in the absence and in the presence of partial ganglionic blockade. During the control study the change in stroke volume index ranged from − 11 to + 10 ml per m^2 and averaged − 1 ml per m^2, representing a mean decrease of 3 per cent. In the presence of partial ganglionic blockade the increase in the stroke volume index ranged from 9.3 to 28 ml per m^2 and averaged 18 ml per m^2, representing a mean increase of 53 per cent. In each subject the stroke volume index rose to a greater extent when transfusion was carried out in the presence of partial ganglionic blockade than in its absence. This difference in response to transfusion was statistically significant ($p < 0.01$).

In the control study before transfusion, the systolic arterial pressure ranged from 90 to 156 mm Hg with an average of 118 mm Hg; the diastolic arterial pressure ranged from 53 to 78 mm Hg with an average of 61 mm Hg. Following transfusion in the control study, the systolic arterial pressure ranged from 117 to 176 mm Hg and averaged 143 mm Hg; the diastolic arterial pressure ranged from 62 to 86 mm Hg and averaged 76 mm Hg.

In the study carried out during Arfonad infusion, before transfusion the systolic arterial pressure ranged from 71 to 95 mm Hg and averaged 84 mm Hg; the diastolic arterial pressure ranged from 39 to 57 mm Hg and averaged 49 mm Hg. Following the transfusion in the studies performed during Arfonad infusion, the systolic arterial pressure ranged from 107 to 151 mm Hg and averaged 128 mm Hg; the diastolic arterial pressure ranged from 65 to 92 mm Hg with an average of 75 mm Hg.

The change in mean systemic arterial pressure induced by transfusion in the absence and in the presence of partial ganglionic blockade is illustrated in Figure 4. During the control study the increment in mean systemic arterial pressure ranged from 5 to 39 mm Hg and averaged 18 mm Hg, representing a mean increase of 23 per cent. In the presence of partial ganglionic blockade the increase in mean systemic arterial pressure ranged

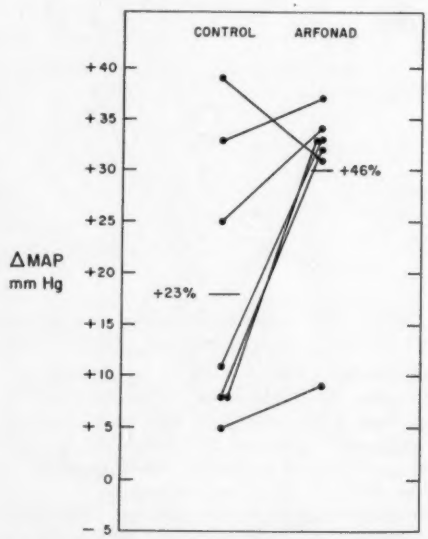

FIG. 4. EFFECT OF ACUTE HYPERVOLEMIA ON MEAN SYSTEMIC ARTERIAL PRESSURE.

from 9 to 37 mm Hg and averaged + 30 mm Hg, representing a mean increase of 46 per cent. In six of the seven subjects the arterial pressure rose more in the presence of partial ganglionic blockade than in its absence. This difference in response to the transfusion was statistically significant ($p < 0.15$).

Figure 5 depicts the change in left ventricular minute work induced by the transfusion in the control study and in the presence of partial ganglionic blockade. During the control study the change in left ventricular minute work ranged from − 0.39 to + 3.43 kg-m per minute per m^2 and averaged + 1.31 kg-m per minute per m^2, representing a mean increase of 37 per cent. In the presence of partial ganglionic blockade the increase in left ventricular minute work ranged from 2.19 to 4.09 kg-m per minute per m^2 and averaged 3.03 kg-m per minute per m^2, representing a mean increase of 111 per cent. In each subject the left ventricular minute work rose to a greater extent when transfusion was carried out in the presence of partial ganglionic blockade than in its absence. The difference in response to transfusion was statistically significant ($p < 0.01$).

Figure 6 illustrates the change in left ventricular stroke work induced by the transfusion in the control study and in the presence of partial ganglionic blockade. During the control study the change in left ventricular stroke work ranged from − 9.9 to + 39.5 g-m per m^2 and averaged + 12.6 g-m per m^2, representing a mean increase of 20 per cent. In the presence of partial ganglionic blockade the change in left ventricular stroke work ranged from + 29 to 58.4 g-m per m^2 and averaged 39.16 g-m per m^2, representing a mean increase of 108 per cent. In each subject the left ventricular stroke work rose to a greater extent when transfusion was carried out in the presence of partial ganglionic blockade than in its absence. The difference in response to transfusion was statistically significant ($p < 0.01$).

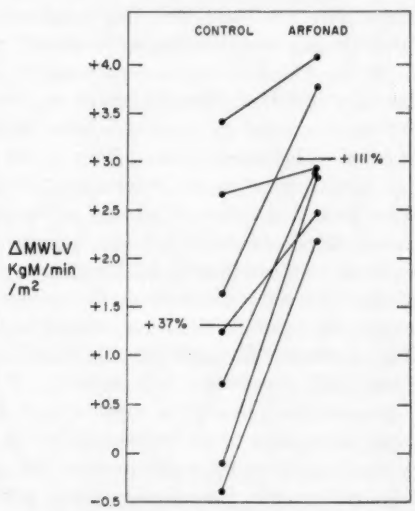

FIG. 5. EFFECT OF ACUTE HYPERVOLEMIA ON LEFT VENTRICULAR MINUTE WORK.

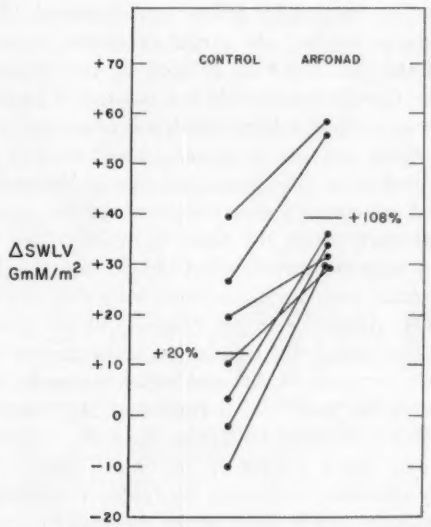

FIG. 6. EFFECT OF ACUTE HYPERVOLEMIA ON LEFT VENTRICULAR STROKE WORK.

DISCUSSION

The response of cardiac output to transfusion in the control studies reported herein is in agreement with the findings of those investigators (2–4, 6, 7) who have observed that the cardiac output is neither increased consistently by large intravenous infusions, nor decreased by venesection of 300 to 900 ml or by the trapping of blood in the extremities (16). In the absence of Arfonad infusion, the large transfusion was not associated with a significant increase in central blood volume. Failure of the central blood volume to increase following large infusions of dextran was also

noted in the dog by Milnor and Bertrand (17). In the presence of the partial ganglionic blockade and the vasodilatation induced by the Arfonad, blood transfusion resulted in a substantial increase in central blood volume which was associated with a striking increase in the output and work of the left ventricle. The important role of the central blood volume as a determinant of cardiac output, particularly when the latter is initially low, has been suggested previously (18, 19) and the data presented herein are consistent with this concept.

This difference in the response of the central blood volume to the transfusion in the absence and in the presence of Arfonad infusion may help to explain the failure of a consistent augmentation of the cardiac output following the acute expansion of total blood volume in the intact state. One may speculate that during the "control" portion of this study and in experiments reported by others (2–4, 6, 7, 17), this expansion of blood volume increased intravascular pressure. The consequent stimulation of arterial (20) and systemic venous baroreceptors (21) could have resulted in a decrease in venous tone resulting in an increased capacity of the venous bed at any given transmural pressure (21–24). This decrease in venous tone, in turn, could result in a tendency for the infused fluid to pool in the systemic venous bed, which would minimize any augmentation of the volume of blood in the heart and lungs (25). Accordingly, a smaller increase in cardiac output and ventricular work occurs than would be expected in the absence of such reflex venodilatation. However, since no measurements of peripheral venous pressure were carried out, this explanation must be considered to be speculative. Furthermore, it is now clear from the experiments of Daly and Luck (26) and of Sarnoff and co-workers (27) that carotid sinus stimulation depresses ventricular function strikingly and thereby diminishes the external work performed by the ventricle from any given filling pressure. In addition, the latter workers have also shown that baroreceptor stimulation depresses atrial contractility. In this manner the contribution of atrial contraction to ventricular filling is diminished and the ventricular stroke work for any given level of mean atrial pressure is further decreased (28). Finally, by inducing reflex arteriolar dilatation, baroreceptor stimulation would tend to diminish further any increase in ventricular work induced by hypervolemia.

It was observed, however, that during the infusion of Arfonad, the blood transfusion resulted in substantial augmentation of the output and work of the left ventricle. It is postulated that as a consequence of the partial ganglionic blockade induced by Arfonad, venous tone diminished, the capacity of the venous bed increased and the central blood volume, cardiac output, and left ventricular work fell. However, it would appear likely from the observations of Alexander (22), that when venous tone diminishes, for any given increment in venous blood volume the transmural venous pressure rises to a greater extent than when the venous bed is initially partially constricted. This would permit a larger fraction of the infused blood to enter the thorax and cardiac output to rise significantly. The concept that even small changes in venous tone may induce striking changes in cardiac output has previously been stressed by Folkow (29).

Pertinent to this possible explanation of the data obtained are those studies which have demonstrated that venous tone in man is not constant, but may be altered by a variety of stimuli (30–34). In particular, ganglionic blockade produces venodilatation in patients in congestive heart failure (35, 36) and in normal subjects during muscular exercise (34). The experiments of Freis and associates have also suggested that hexamethonium blocks reflex venoconstriction in normal subjects (37). These workers demonstrated that phlebotomies of 250 to 500 ml resulted in a striking decline in arterial pressure only after the administration of hexamethonium. This would appear to be analogous to the observation (Figure 4) that a greater elevation of arterial pressure occurs when blood was infused in the presence of the Arfonad infusion than in its absence.

Although the subjects were mildly anemic in the present study, whole blood was utilized instead of saline, dextran, plasma or albumin in order to avoid any acute change in the hematocrit. It has been demonstrated clearly in anesthetized dogs that acute depression of the hematocrit by the dilution resulting from large intravenous infusions of fluid, rather than from hypervolemia per se, may be responsible for the elevation in cardiac output noted when hemodilution occurs (38–40).

Although there was a significant lowering of hematocrit by the phlebotomies prior to the initial study, there were no striking differences between the hematocrits at the beginning and completion of each transfusion, nor between those obtained in the course of the first and second studies. The direct effects of partial ganglionic blockade on myocardial function are not considered likely to account for the experimental results obtained, since it has been demonstrated (41) that hexamethonium actually depresses myocardial contractility.

Experiments in which hypervolemia is induced acutely in intact human subjects may not be ideally suited for determining the applicability of Starling's law of the heart to man. The presence of an intact autonomic nervous system results in failure of a significant fraction of the infused fluids to augment the volume of blood within the heart and lungs, and the stimulation of myocardial contraction provided by an increased end-diastolic fiber length cannot result (42). Starling's law of the heart in man may, perhaps, be examined more profitably by studying the relationship between ventricular filling pressure or fiber length and ventricular stroke work under conditions in which significant variations in filling are induced. Such studies are now in progress.

SUMMARY

1. The cardiovascular response to transfusion in the absence and presence of Arfonad has been studied in seven human subjects with normal cardiovascular systems.

2. Transfusion in the control state produced no significant change in central blood volume, an average elevation of cardiac output of 0.50 ± 1.37 L per minute and of left ventricular stroke work of 23.3 ± 33.8 g-m. The increase in each of these parameters resulting from transfusion during partial ganglionic blockade was substantially greater and averaged 348 ± 188 ml, 2.40 ± 0.61 L per minute, and 73.3 ± 25.7 g-m, respectively.

3. It is suggested that when hypervolemia is induced acutely in intact man marked alterations in circulatory dynamics are prevented by the activity of the autonomic nervous system, which results in reflex venodilatation and depression of myocardial contractility. Accordingly, Starling's law of the heart cannot be readily demonstrated. When hypervolemia is induced after the activity of the autonomic nervous system has been reduced, more striking hemodynamic changes occur, resembling those noted in the Starling heart-lung preparation when venous inflow is augmented.

REFERENCES

1. McMichael, J., and Sharpey-Schafer, E. P. Cardiac output in man by a direct Fick method. Effects of posture, venous pressure change, atropine and adrenaline. Brit. Heart J. 1944, **6**, 33.
2. Warren, J. V., Brannon, E. S., Weens, H. S., and Stead, E. A., Jr. Effect of increasing the blood volume and right atrial pressure on the circulation of normal subjects by intravenous infusions. Amer. J. Med. 1948, **4**, 193.
3. Doyle, J. T., Wilson, J. S., Estes, E. H., and Warren, J. V. The effect of intravenous infusions of physiologic saline solution on the pulmonary arterial and pulmonary capillary pressure in man. J. clin. Invest. 1951, **30**, 345.
4. Schnabel, T. G., Jr., Eliasch, H., Thomasson, B., and Werkö, L. The effect of experimentally induced hypervolemia on cardiac function in normal subjects and patients with mitral stenosis. J. clin. Invest. 1959, **38**, 117.
5. Witham, A. C., Fleming, J. W., and Bloom, W. L. The effect of intravenous administration of dextran on cardiac output and other circulatory dynamics. J. clin. Invest. 1951, **30**, 897.
6. Fleming, J. W., and Bloom, W. L. Further observations on the hemodynamic effect of plasma volume expansion by dextran. J. clin. Invest. 1957, **36**, 1233.
7. Stead, E. A., Jr., Brannon, E. S., Merrill, A. J., and Warren, J. V. Concentrated human albumin in the treatment of shock. Arch. intern. Med. 1946, **77**, 564.
8. Randall, L. O., Peterson, W. G., and Lehmann, G. The ganglionic blocking action of thiophanium derivatives. J. Pharmacol. exp. Ther. 1949, **97**, 48.
9. McCubbin, J. W., and Page, I. H. Nature of the hypotensive action of a thiophanium derivative (RO 2-2222) in dogs. J. Pharmacol. exp. Ther. 1952, **105**, 437.
10. Hamilton, W. F., Moore, J. W., Kinsman, J. M., and Spurling, R. G. Studies on the circulation. IV. Further analysis of the injection method and of changes in hemodynamics under physiological and pathological conditions. Amer. J. Physiol. 1932, **99**, 534.
11. Shadle, O. W., Ferguson, T. B., Gregg, D. E., and Gilford, S. R. Evaluation of a new cuvette densi-

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Although the subjects were mildly anemic in the present study, whole blood was utilized instead of saline, dextran, plasma or albumin in order to avoid any acute change in the hematocrit. It has been demonstrated clearly in anesthetized dogs that acute depression of the hematocrit by the dilution resulting from large intravenous infusions of fluid, rather than from hypervolemia per se, may be responsible for the elevation in cardiac output noted when hemodilution occurs (38–40).

Although there was a significant lowering of hematocrit by the phlebotomies prior to the initial study, there were no striking differences between the hematocrits at the beginning and completion of each transfusion, nor between those obtained in the course of the first and second studies. The direct effects of partial ganglionic blockade on myocardial function are not considered likely to account for the experimental results obtained, since it has been demonstrated (41) that hexamethonium actually depresses myocardial contractility.

Experiments in which hypervolemia is induced acutely in intact human subjects may not be ideally suited for determining the applicability of Starling's law of the heart to man. The presence of an intact autonomic nervous system results in failure of a significant fraction of the infused fluids to augment the volume of blood within the heart and lungs, and the stimulation of myocardial contraction provided by an increased end-diastolic fiber length cannot result (42). Starling's law of the heart in man may, perhaps, be examined more profitably by studying the relationship between ventricular filling pressure or fiber length and ventricular stroke work under conditions in which significant variations in filling are induced. Such studies are now in progress.

SUMMARY

1. The cardiovascular response to transfusion in the absence and presence of Arfonad has been studied in seven human subjects with normal cardiovascular systems.

2. Transfusion in the control state produced no significant change in central blood volume, an average elevation of cardiac output of 0.50 ± 1.37 L per minute and of left ventricular stroke work of 23.3 ± 33.8 g-m. The increase in each of these parameters resulting from transfusion during partial ganglionic blockade was substantially greater and averaged 348 ± 188 ml, 2.40 ± 0.61 L per minute, and 73.3 ± 25.7 g-m, respectively.

3. It is suggested that when hypervolemia is induced acutely in intact man marked alterations in circulatory dynamics are prevented by the activity of the autonomic nervous system, which results in reflex venodilatation and depression of myocardial contractility. Accordingly, Starling's law of the heart cannot be readily demonstrated. When hypervolemia is induced after the activity of the autonomic nervous system has been reduced, more striking hemodynamic changes occur, resembling those noted in the Starling heart-lung preparation when venous inflow is augmented.

REFERENCES

1. McMichael, J., and Sharpey-Schafer, E. P. Cardiac output in man by a direct Fick method. Effects of posture, venous pressure change, atropine and adrenaline. Brit. Heart J. 1944, **6**, 33.
2. Warren, J. V., Brannon, E. S., Weens, H. S., and Stead, E. A., Jr. Effect of increasing the blood volume and right atrial pressure on the circulation of normal subjects by intravenous infusions. Amer. J. Med. 1948, **4**, 193.
3. Doyle, J. T., Wilson, J. S., Estes, E. H., and Warren, J. V. The effect of intravenous infusions of physiologic saline solution on the pulmonary arterial and pulmonary capillary pressure in man. J. clin. Invest. 1951, **30**, 345.
4. Schnabel, T. G., Jr., Eliasch, H., Thomasson, B., and Werkö, L. The effect of experimentally induced hypervolemia on cardiac function in normal subjects and patients with mitral stenosis. J. clin. Invest. 1959, **38**, 117.
5. Witham, A. C., Fleming, J. W., and Bloom, W. L. The effect of intravenous administration of dextran on cardiac output and other circulatory dynamics. J. clin. Invest. 1951, **30**, 897.
6. Fleming, J. W., and Bloom, W. L. Further observations on the hemodynamic effect of plasma volume expansion by dextran. J. clin. Invest. 1957, **36**, 1233.
7. Stead, E. A., Jr., Brannon, E. S., Merrill, A. J., and Warren, J. V. Concentrated human albumin in the treatment of shock. Arch. intern. Med. 1946, **77**, 564.
8. Randall, L. O., Peterson, W. G., and Lehmann, G. The ganglionic blocking action of thiophanium derivatives. J. Pharmacol. exp. Ther. 1949, **97**, 48.
9. McCubbin, J. W., and Page, I. H. Nature of the hypotensive action of a thiophanium derivative (RO 2-2222) in dogs. J. Pharmacol. exp. Ther. 1952, **105**, 437.
10. Hamilton, W. F., Moore, J. W., Kinsman, J. M., and Spurling, R. G. Studies on the circulation. IV. Further analysis of the injection method and of changes in hemodynamics under physiological and pathological conditions. Amer. J. Physiol. 1932, **99**, 534.
11. Shadle, O. W., Ferguson, T. B., Gregg, D. E., and Gilford, S. R. Evaluation of a new cuvette densi-

tometer for determination of cardiac output. Circulat. Res. 1953, **1**, 200.

12. Gilford, S. R., Gregg, D. E., Shadle, O. W., Ferguson, T. B., and Marzetta, L. An improved cuvette densitometer for cardiac output determination by the dye-dilution method. Rev. Scient. Inst. 1953, **24**, 696.
13. Sabiston, D. C., Jr., Khouri, E. M., and Gregg, D. E. Use and application of the cuvette densitometer as an oximeter. Circulat. Res. 1957, **5**, 125.
14. Braunwald, E., Fishman, A. P., and Cournand, A. Estimation of the volume of a circulatory model by the Hamilton and Bradley methods at varying flow volume ratios. J. appl. Physiol. 1958, **12**, 445.
15. Braunwald, E., Binion, J. T., Morgan, W. L., Jr., and Sarnoff, S. J. Alterations in central blood volume and cardiac output induced by positive pressure breathing and counteracted by Metaraminol (Aramine). Circulat. Res. 1957, **5**, 670.
16. Warren, J. V., Brannon, E. S., Stead, E. A., Jr., and Merrill, A. J. The effect of venesection and pooling of blood in the extremities on the atrial pressure and cardiac output in normal subjects with observations on acute circulatory collapse in three instances. J. clin. Invest. 1945, **24**, 337.
17. Milnor, W. R., and Bertrand, C. A. Estimation of venous blood volume in the dog by the indicator-dilution method. Circulat. Res. 1958, **6**, 55.
18. Sjöstrand, T. The regulation of the blood distribution in man. Acta physiol. scand. 1952, **26**, 312.
19. Warren, J. V., Weissler, A. M., and Leonard, J. J. Observations on the determinants of cardiac output. Trans. Ass. Amer. Phycns 1957, **70**, 268.
20. Heymans, C., and Neil, E. Reflexogenic Areas of the Cardiovascular System. Boston, Little, Brown and Company, 1958.
21. Alexander, R. S. Reflex alterations in venomotor tone produced by venous congestion. Circulat. Res. 1956, **4**, 49.
22. Alexander, R. S. The participation of the venomotor system in pressor reflexes. Circulat. Res. 1954, **2**, 405.
23. Salzman, E. W. Reflex peripheral venoconstriction induced by carotid occlusion. Circulat. Res. 1957, **5**, 149.
24. Sarnoff, S. J. Some physiologic considerations in the genesis of acute pulmonary edema *in* Pulmonary Circulation, W. R. Adams and I. Veith, Eds. New York, Grune and Stratton, 1959, pp. 273–282.
25. Rashkind, W. J., Lewis, D. H., Henderson, J. B., Heiman, D. F., and Dietrick, R. B. Venous return as affected by cardiac output and total peripheral resistance. Amer. J. Physiol. 1953, **175**, 415.
26. Daly, M. deB., and Luck, C. P. The effects of carotid sinus baroreceptor reflexes on pulmonary arterial pressure and pulmonary blood flow in the dog. J. Physiol. 1958, **143**, 343.
27. Sarnoff, S. J., Brockman, S. K., Gilmore, J. P., Mitchell, J., and Linden, R. J. The influence of carotid sinus pressure on myocardial contractility. Fed. Proc. 1959, **18**, 137.
28. Brockman, S. K., Linden, R. J., Mitchel, J., Gilmore, J. P., and Sarnoff, S. J. The effect of vagal stimulation on ventricular function. Fed. Proc. 1959, **18**, 17.
29. Folkow, B. Nervous control of the blood vessels. Physiol. Rev. 1955, **35**, 629.
30. Wood, J. E., Litter, J., and Wilkins, R. W. Peripheral venoconstriction in human congestive heart failure. Circulation 1956, **13**, 524.
31. Wood, J. E., and Eckstein, J. W. A tandem forearm plethysmograph for study of acute responses of the peripheral veins of man: The effect of environmental and local temperature change, and the effect of pooling blood in the extremities. J. clin. Invest. 1958, **37**, 41.
32. Eckstein, J. W., Hamilton, W. K., and McCammond, J. M. Pressure-volume changes in the forearm veins of man during hyperventilation. J. clin. Invest. 1958, **37**, 956.
33. Eckstein, J. W., and Hamilton, W. K. The effects of isoproterenol on peripheral venous tone and transmural right atrial pressure in man. J. clin. Invest. 1959, **38**, 342.
34. Merritt, F. L., and Weissler, A. M. Reflex venomotor alterations during exercise and hyperventilation. Amer. Heart J. 1959, **58**, 382.
35. Burch, G. E., and Murtadha, M. A study of the venomotor tone in a short intact venous segment of the forearm of man. Amer. Heart J. 1956, **51**, 807.
36. Burch, G. E. A method for measuring venous tone in digital veins of intact man. A.M.A. Arch. intern. Med. 1954, **94**, 724.
37. Freis, E. D., Stanton, J. R., Finnerty, F. A., Schnaper, H. W., Johnson, R. L., Rath, C. E., and Wilkins, R. W. The collapse produced by venous congestion of the extremities or by venesection following certain hypotensive agents. J. clin. Invest. 1951, **30**, 435.
38. Sunahara, F. A., and Beck, L. Cardiovascular effect of acutely produced anemia in the normal dog. Amer. J. Physiol. 1954, **176**, 139.
39. Sunahara, F. A., Hatcher, J. D., Beck, L., and Gowdly, C. W. Cardiovascular responses in dogs to intravenous infusions of whole blood, plasma, and plasma followed by packed erythrocytes. Canad. J. Biochem 1955, **33**, 349.
40. Fowler, N. O., Bloom, W. L., and Ward, J. A. Hemodynamic effects of hypervolemia with and without anemia. Circulat. Res. 1958, **6**, 163.
41. Cotten, M. deV., Brown, J. M., and Kronen, P. S. Heart force responses to pressor amines during hypotension produced by hexamethonium. Anesthesiology 1954, **15**, 126.
42. Starling, E. H. The Linacre Lecture on the Law of the Heart (Cambridge, 1915). London, Longmans, Green and Company, 1918.

THE EFFECT OF CHANGES IN POSTURE AND OF GRADED EXERCISE ON STROKE VOLUME IN MAN *

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Interest has been revived recently in the relative importance of changes in heart rate and stroke volume during exercise in man. Rushmer and Smith have examined the literature and concluded that an increase in stroke volume is not an essential feature of the ventricular response in normal human subjects (1, 2). Contrary views, particularly in the older literature, were attributed to differences in the technics used to measure cardiac output and in the degree of training of the subjects (1). However, in the studies reviewed by Rushmer, the subjects had exercised in various postures. This could have accounted for much of the apparent discrepancy (3), a point which Rushmer has now emphasized (4).

The present investigation demonstrates the importance of the posture of the subject in the interpretation of changes in stroke volume from rest to exercise. The stroke volume increases only slightly once exercise has begun, regardless of the severity of the exercise or the position assumed by the subject.

METHODS

Seven healthy "untrained" men, aged 25 to 35 years, were studied. Cardiac output was measured by the indicator-dilution method. A cardiac catheter was placed with its tip in the superior vena cava. The indicator, cardio-green dye,[1] was given in a dose of 7.5 mg by sudden injection from a specially calibrated syringe. The syringe was attached to the catheter throughout the experiment and was filled before each injection from a supply syringe connected to it by a three-way tap. Blood was sampled continuously for each dilution curve from the radial artery through a Wood oximeter at rates of 28 to 50 ml per minute. The dilution curve was recorded on a photokymograph. Not more than 40 ml was withdrawn for any curve. On completion of each curve the blood was reinfused.

Samples of blood were withdrawn from the radial artery before and after each experiment. Known amounts of dye were added to 10 ml aliquots of blood and the resulting mixtures were drawn through the cuvet oximeter to permit calibration of the dilution curves. The calibrations for the two samples invariably were close (Figure 1, upper panel) and were reproducible from day to day. Calibrations for blood from different subjects were similar. Figure 1 (lower panel) also shows the mean values and 95 per cent confidence limits for 14 sets of observations from the 4 subjects who performed exercise

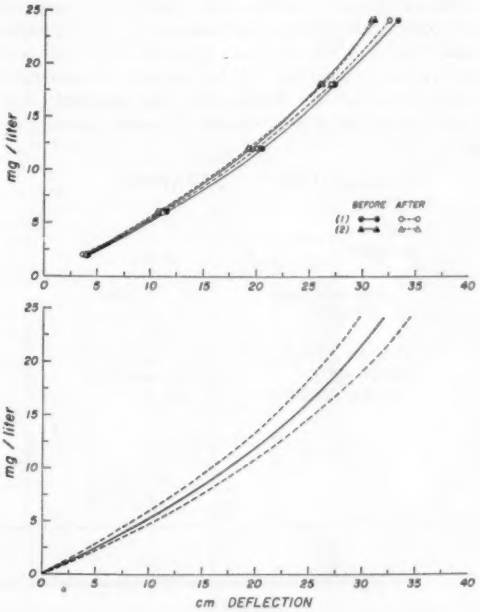

FIG. 1. CALIBRATIONS OF DYE-BLOOD MIXTURES FOR SAMPLES TAKEN BEFORE AND AFTER A COMPLETE EXPERIMENT ON EACH OF TWO OCCASIONS IN R.M. (UPPER PANEL); MEAN VALUES AND 95 PER CENT CONFIDENCE LIMITS OF CALIBRATIONS FOR 14 BLOOD SAMPLES IN SIX SUBJECTS (LOWER PANEL). The same cuvet oximeter was used on every occasion.

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† Minnesota Heart Association, Fellow in Physiology, Mayo Foundation.

‡ The Mayo Foundation, Rochester, Minnesota, is a part of the Graduate School of the University of Minnesota.

[1] Kindly supplied by Hynson, Westcott and Dunning, Baltimore, Md.

in the upright position and from 2 additional subjects, the same cuvet oximeter being used on each occasion.

The heart rate was recorded by an electrocardiograph and a cardiotachometer. Radial artery pressure was recorded continuously except during inscription of the indicator-dilution curves.

In some experiments expired air was collected in a neoprene bag for 5 minutes with the subject at rest and for periods of 1 or 2 minutes during each period of exercise. Low resistance valves and tubing were used. The oxygen consumption so measured was used only to provide an index of energy expenditure. Surface area was determined from each subject's height and weight by the nomogram of Dubois and Dubois (5).

In 9 experiments observations were made first on 4 subjects at rest in the supine position. The observations were then repeated after the subject stood for 15 to 20 minutes in a relaxed manner. This was followed by mild leg exercise, consisting of alternate contraction and relaxation of the calf muscle or marking time. Subsequent measurements were made with the subject walking up a treadmill, tilted at 12 degrees from the horizontal, at 1.7, 2.5, 3.5, and 4.5 miles per hour (mph). Successive series of exercises were separated by periods of 10 to 15 minutes during which the subject stood at ease or gently contracted his calf muscles. The right forearm and hand were fastened to a horizontal board at, or slightly below, heart level and the left hand gripped the board. The zero reference point for arterial pressure measurements was taken as halfway between the front and back of the thorax while the subject was in the supine position and at the level of the third costal cartilage while he was standing or walking. At each exercise the indicator-dilution curve was recorded during the third minute.

Cardiac output and heart rate were measured during the performance of leg exercise in 4 supine subjects. Two of the subjects in further experiments walked on the treadmill at 3.5 mph for 10 to 11 minutes. During this period the heart rate was monitored continuously and indicator-dilution curves were obtained at intervals.

RESULTS

Indicator-dilution curves obtained in the first experiment on Subject Y.W. are shown in Figure 2. With the subject standing at rest the cardiac output was 5.7 L per minute, the heart rate 98 beats per minute, and the stroke volume 58 ml. When the subject walked at 3.5 mph up the inclined treadmill the cardiac output increased to 18.8 L per minute and the heart rate to 164 beats per minute; the stroke volume (115 ml) was doubled.

Data from the nine experiments on the four subjects who performed exercise in the upright position are shown in Figure 3 and summarized in Table I. During rest in the supine position the cardiac output was between 6.1 and 7.5 L per min-

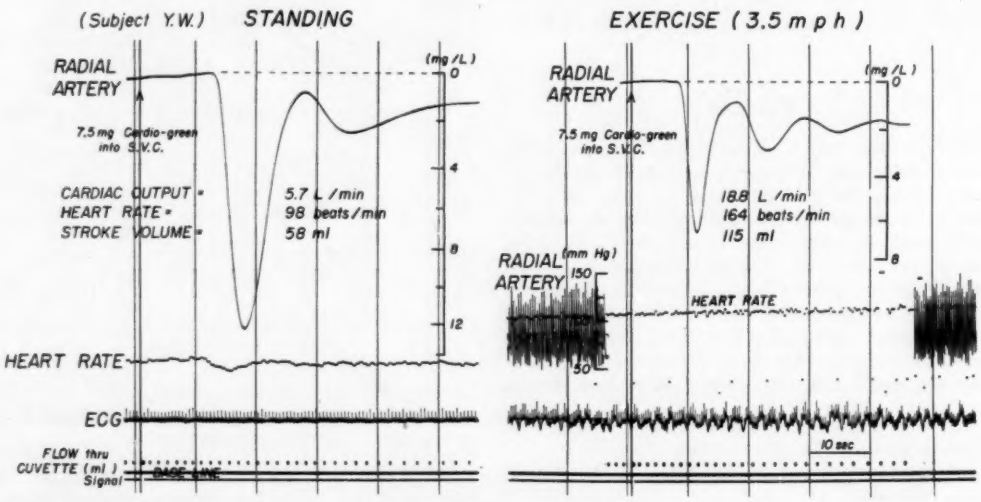

FIG. 2. INDICATOR-DILUTION CURVES OBTAINED WITH THE SUBJECT STANDING AT REST (LEFT PANEL) AND WALKING AT 3.5 MPH ON THE INCLINED TREADMILL (RIGHT PANEL). The calibrations are shown on the right. The vertical lines with arrow heads indicate the time of injection. Beneath are shown the cardiotachometer record of heart rate, the electrocardiogram, and the interrupted line signaling the passage of each milliliter of blood through the cuvet. In the right panel the radial artery pressure also is shown; the instability of the baseline in the electrocardiogram is due to the rapid deep respirations.

TABLE I

Changes in cardiac output, pulse rate, stroke volume, oxygen consumption and blood pressure during upright exercise

Subj. BSA		Position		Light exercise*		Walking speed (mph)			
		Supine	Standing	A	B	1.7	2.5	3.5	4.5
D.W. (1.91 m²)	[1] C.O.	7.2	6.8			14.7	17.5	20.3	
	P.R.	63	83			128	150	170	
	S.V.	114	82			115	117	119	
	O_2	280				1,500	1,970	2,380	
	B.P.	140/75	125/75			135/75	150/75	165/80	
	[2] C.O.	6.7	4.1			11.3	16.7	16.6	
	P.R.	68	87			116	141	157	
	S.V.	98	47			97	118	106	
	B.P.	120/70	120/80			150/75	160/80	160/85	
	[3] C.O.	7.5	4.0	7.1		16.1	17.2	21.6	20.0
	P.R.	72	90	87		130	132	168	174
	S.V.	104	44	82		124	130	129	114
	O_2	280	305	390					
	B.P.	135/75	135/75	135/70		140/65	140/65	155/75	160/75
R.M. (2.10 m²)	[1] C.O.	6.9	6.2			14.3	20.7		25.6
	R.P.	66	87			133	170		180
	S.V.	104	71			108	122		143
	O_2	300				1,530	2,410		2,820
	B.P.	130/65	115/65			175/65	185/65		180/65
	[2] C.O.	6.5	7.1					20.0	20.9
	P.R.	58	75					165	177
	S.V.	111	94					121	118
	B.P.	115/65	130/75					200/70	200/70
	[3] C.O.	7.4	5.8	8.0	12.2		15.5	20.0	22.3
	R.P.	63	82	80	104		158	177	183
	S.V.	117	71	100	117		98	113	122
	O_2	320	340	490	1,000				
	B.P.	115/65	120/70	150/70	145/65		170/70	200/75	205/75
Y.W. (1.85 m²)	[1] C.O.	6.6	5.7			11.1	12.7	18.8	20.2
	P.R.	68	98			120	132	164	174
	S.V.	97	58			93	96	115	116
	O_2	280	260			1,240	1,360	1,840	2,150
	B.P.	120/70	105/70			120/50	115/55	125/60	140/70
	[2] C.O.	6.1	4.6	7.3				19.2	
	P.R.	65	84	80				165	
	S.V.	93	55	92				116	
	O_2		300	360					
	B.P.	120/65	110/65	120/65				130/60	
D.H. (1.73 m²)	[1] C.O.	6.2	4.6			8.8	12.1	15.3	14.9
	P.R.	65	89			96	126	166	168
	S.V.	96	52			92	95	92	89
	O_2	260	270			1,080	1,640	2,030	1,840
	B.P.	130/60	115/55			140/55	140/50	155/55	165/55

* A = contraction and relaxation of calf muscles; B = marking time.

ute. The average cardiac index for all observations was 3.7 L in D.W., 3.3 L in R.M., and 3.4 L in Y.W.; the cardiac index was 3.6 L in D.H. for a single observation. The average cardiac index for all subjects was 3.5 L. The pulse rates were from 58 to 72 beats per minute, the systemic blood pressure from 115/65 to 140/75 mm of mercury, and the stroke volume from 93 to 117 ml. Table II shows the stroke index which ranged from 51 to 56 ml (average, 54 ml) and was similar from

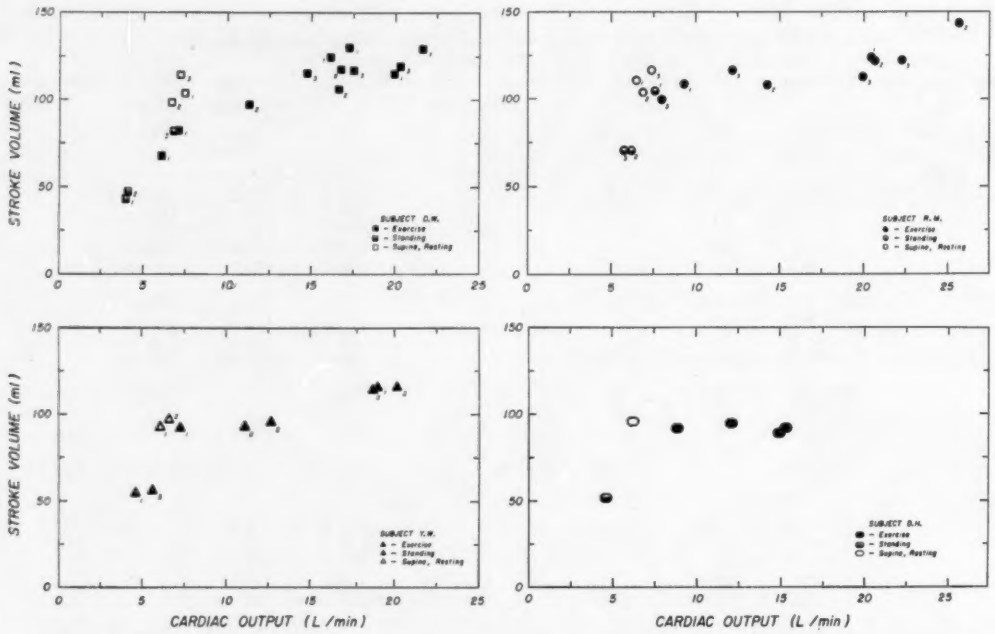

FIG. 3. CHANGES IN STROKE VOLUME AND CARDIAC OUTPUT IN EACH OF THE FOUR SUBJECTS. White symbols, data for subjects resting in supine position; black and white symbols, data while standing; black symbols, data at different grades of treadmill exercise. Numerals adjacent to the symbols indicate the particular experiment from which data were obtained (Table I).

experiment to experiment in each subject (Figure 3, Table I).

On standing there was an increase in heart rate, little change in systemic blood pressure, and, on eight of the nine occasions, a decrease in cardiac output. The stroke index fell to 30 ml in three subjects (Table II). In R.M. the somewhat higher average value of 38 ml was due to a slight fall to 46 ml on one occasion when the subject was apprehensive; in each of the other two experiments in R.M. the stroke volume fell to 34 ml. The average stroke index for all subjects while standing at rest was 32 ml. This represents a fall of 41 per cent from the average value of 54 ml in the supine position.

Mild exercise, consisting of alternate contraction and relaxation of the calf muscles, was undertaken by D.W., R.M., and Y.W. The oxygen consumption was increased by less than 50 per cent (average 315 to 415 ml per minute). Cardiac output increased moderately and there was a reduction of 2 to 4 beats per minute in heart rate. Thus the stroke volume increased almost to the level previously obtained in the supine position (Figure 4). Moderate exercise, achieved by walking uphill at 1.7 to 2.5 mph, was associated with a further rise in stroke volume to values similar to those obtained with the subject in the supine position (Tables I and II, Figure 3). Thus, at 1.7 mph the average stroke index for all subjects was 54 ml, and at 2.5 mph it was 56 ml, compared with 54 ml at rest in the supine position. Severe exercise, sufficient to increase the cardiac output to between 15 and 25 L per minute, was accompanied by a further slight though definite rise in stroke volume in the first three subjects (Tables I and II, Figure 3); D.H. did not achieve an output greater than 15 L per minute, and at this level the stroke volume was slightly less than that when he was at rest in the supine position. The average stroke index at 3.5 mph was 58 ml, and at 4.5 mph, 59 ml.

The first three subjects achieved a cardiac output of 20 L per minute or more on at least one

TABLE II

*Stroke index and heart rate at rest and during upright exercise**

Subj.	BSA	Stroke index (ml)							
				Walking speed (mph)				Per cent of change at 4.5 mph above that at rest	
		Supine	Standing	1.7	2.5	3.5	4.5	Supine	Standing
	m^2								
D.W.	1.91	55	30	59	64	62	60	+9	+100
R.M.	2.10	54	38	54	52	56	61	+13	+61
Y.W.	1.85	51	30	50	52	62	63	+23	+110
D.H.	1.73	56	30	53	55	53	51	−9	+70
		Heart rate (beats per minute)							
D.W.	1.91	68	87	125	141	165	174	+156	+100
R.M.	2.10	62	81	119	164	171	180	+190	+122
Y.W.	1.85	66	91	120	132	165	174	+164	+91
D.H.	1.73	65	89	96	126	166	168	+158	+77

* Table shows average values for all experiments in each of three subjects, and values for the single experiment in D.H.

occasion, and heart rates of 174 to 183 beats per minute. The oxygen consumption increased in D.W. to 2,380, in R.M. to 2,820, and in Y.W. to 2,150 ml per minute, in comparison with their respective values of 280, 300, and 280 at rest in the supine position; these increases are 8.5, 9.4, and 7.7 times the values prior to the increase (Figure 5). The levels of exercise were approaching the maximum which the subjects could maintain for the necessary 3 minutes. The fourth subject (D.H.) achieved a maximal output of 15.3 L per minute, a heart rate of 168 beats per minute and an oxygen consumption of 2,030 ml per minute, an increase of 8.0 times the resting value.

Arteriovenous difference in oxygen content was calculated from the oxygen consumption and cardiac output (Figure 6). At rest in the supine position the values were 3.9 to 4.3 ml per 100 ml of blood. With moderate exercise there was an increase to 10 to 12 ml per 100 ml. Little further change occurred in the arteriovenous oxygen difference even with the severest exercise undertaken.

Data from the four subjects who performed leg

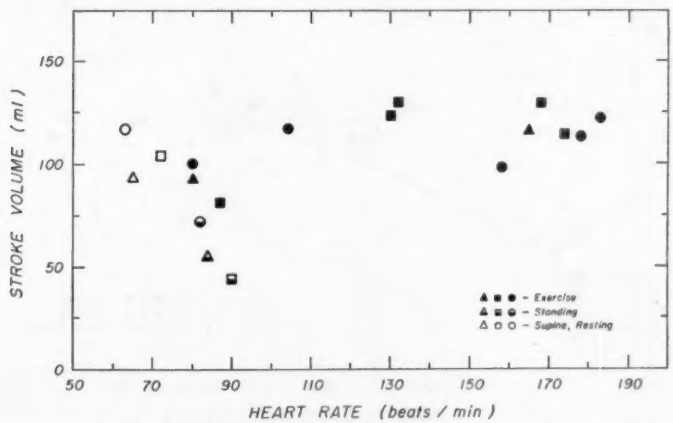

FIG. 4. RELATIONSHIP BETWEEN HEART RATE AND STROKE VOLUME IN ONE EXPERIMENT FROM EACH OF THREE SUBJECTS. Symbols used are the same as in Figure 3. Note the tachycardia and fall in stroke volume with the subject standing, and the slight bradycardia and marked rise in stroke volume on performing light exercise in the upright position.

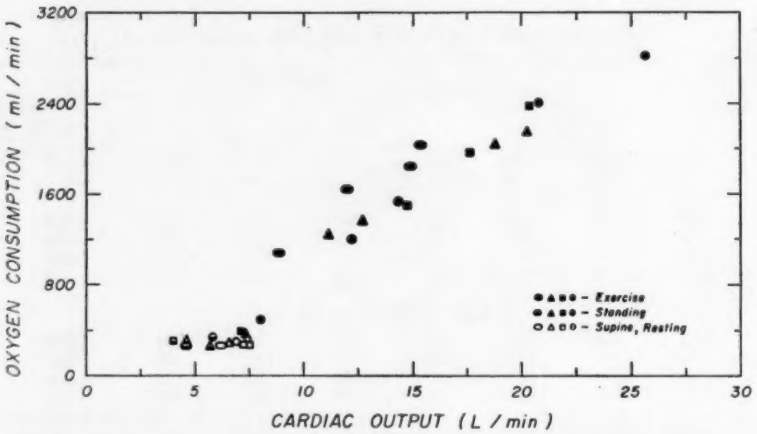

FIG. 5. RELATION BETWEEN CARDIAC OUTPUT AND OXYGEN CONSUMPTION AT REST IN THE SUPINE AND THE STANDING POSITIONS AND DURING EXERCISE. Symbols used are the same as those in Figure 3.

exercise on a cycle ergometer while supine are presented in Table III. The exercise was arbitrarily graded as mild, moderate or severe. There was a definite, though slight, increase in stroke volume during exercise; with the severest exercise the stroke volume was 10 to 20 per cent greater than that while at rest. In Figure 7 the data obtained for Y.W. are compared with those on the other occasions when he exercised in the upright position.

The effects of severe exercise maintained for 10-minute periods are shown in Figure 8. In R.M. the pulse rate increased from 80 to 165 beats per minute within 2 minutes and was steady for the remainder of the exercise; the blood pressure increased from 132/75 to 200/70 mm of mercury and also remained fairly steady for the next 8 minutes. Values obtained for cardiac output were about 20 L per minute at 2 and 3.5 minutes, 17 L per minute at the sixth minute and 19

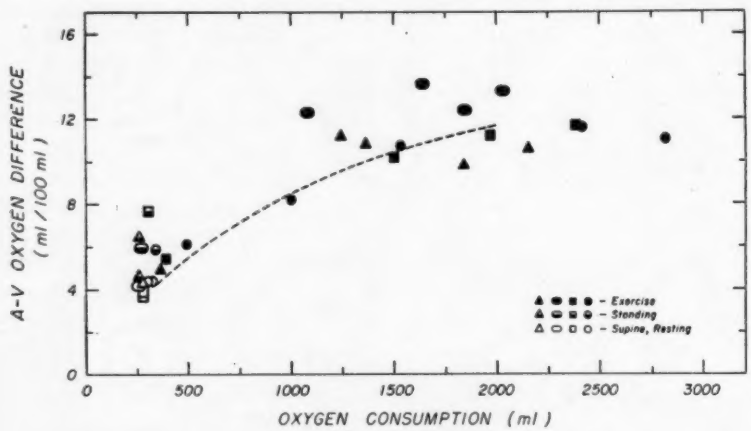

FIG. 6. RELATIONSHIP BETWEEN OXYGEN CONSUMPTION AND ARTERIOVENOUS DIFFERENCE IN OXYGEN CONTENT (ONE EXPERIMENT FROM EACH SUBJECT). The lowest values shown were obtained with subject at rest in the supine position. The interrupted line shows the regression line obtained by Donald and associates (6) plotted for the hypothetical man with a body surface area of 1.73 m^2.

TABLE III

Changes in cardiac output, heart rate and stroke volume during exercise while supine

Subj.	Condition	Cardiac output	Heart rate	Stroke volume	Stroke index
		L/min	*beats/min*	*ml*	*ml*
J.B.	Rest	7.30	73	100	51
	Rest	6.80	71	96	49
	Moderate exercise	12.20	117	104	53
	Moderate exercise	12.60	125	101	52
	Severe exercise	15.00	140	107	55
W.M.	Rest	7.25	57	127	59
	Rest	7.20	57	126	59
	Severe exercise	16.50	115	143	67
	Severe exercise	15.95	113	141	66
H.H.	Rest	8.15	93	88	49
	Rest	7.85	96	82	46
	Moderate exercise	13.90	142	98	54
	Moderate exercise	12.30	132	93	52
Y.W.	Rest	5.35	59	91	49
	Rest	5.65	61	93	50
	Mild exercise	8.70	90	97	52
	Mild exercise	8.75	89	98	53
	Moderate exercise	10.90	100	109	59
	Severe exercise	13.40	120	112	61
	Severe exercise	15.10	138	109	59

L per minute at the tenth minute. In Y.W., the pulse rate increased from 80 to 153 beats per minute at the second minute, but continued to rise slowly throughout the period of exercise, reaching 189 beats per minute at the tenth minute. The systolic blood pressure increased by only 10 mm of mercury; the diastolic pressure initially fell from 75 to about 60 mm of mercury and then gradually increased to 70 mm of mercury at the tenth minute. The cardiac output was 7.2 L per minute with the subject standing at rest; 19 L at 3.5 minutes of exercise, 22.5 L at 6 minutes, and 25 L at the tenth minute; corresponding values for stroke volume were 93, 116, 130, and 133 ml.

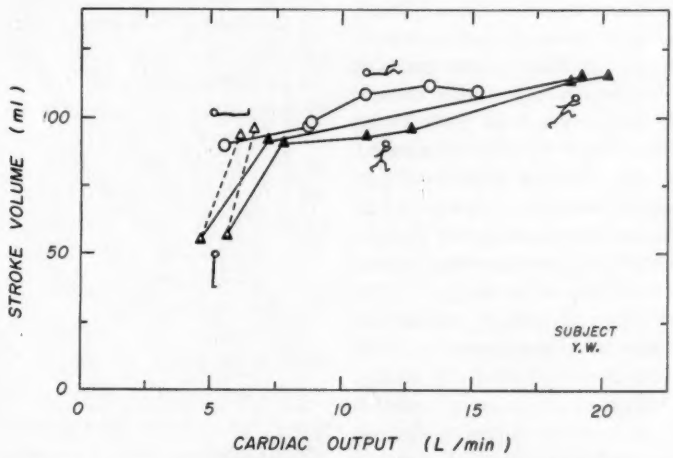

FIG. 7. CHANGES IN STROKE VOLUME IN Y.W. DURING LEG EXERCISE WHILE SUPINE (WHITE CIRCLES), COMPARED WITH DATA OBTAINED WITH SUBJECT AT REST AND DURING EXERCISE IN THE UPRIGHT POSITION. Symbols as for Y.W. in Figure 3.

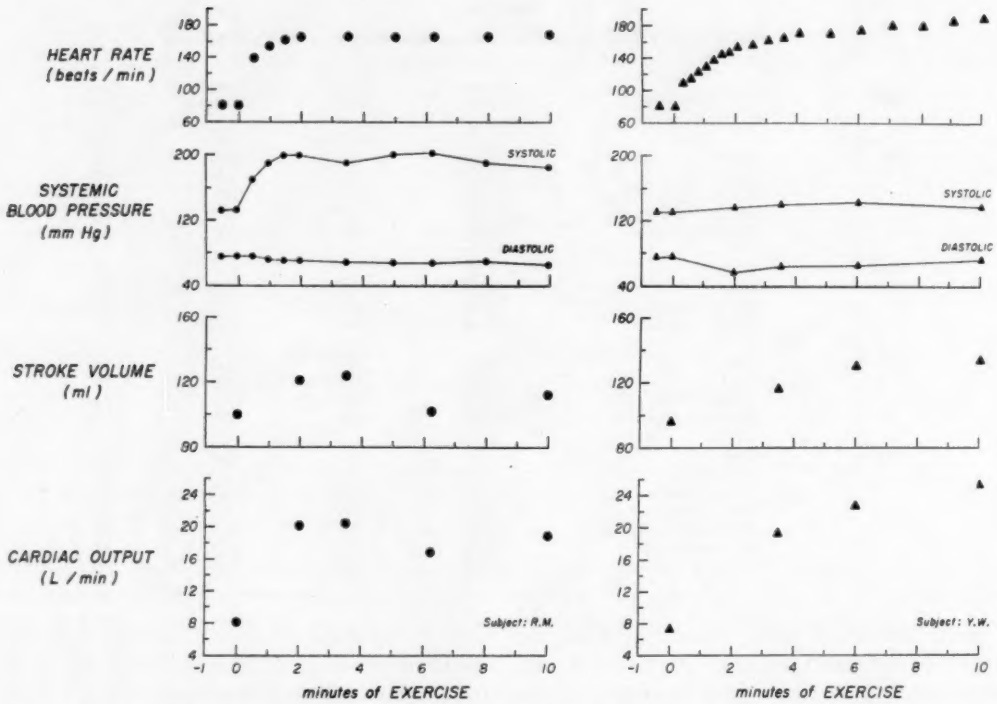

FIG. 8. THE EFFECT OF CONTINUED EXERCISE WHILE UPRIGHT IN R.M. (LEFT PANEL) AND Y.W. (RIGHT PANEL) ON HEART RATE, BLOOD PRESSURE, STROKE VOLUME AND CARDIAC OUTPUT.

DISCUSSION

Donald, Bishop, Cumming and Wade (6) found that during supine leg exercise the arteriovenous oxygen difference and cardiac output reach a steady state after the first minute. In the present experiments, pulse rate (except in Y.W.) and blood pressure became stable in 1.5 to 2 minutes and cardiac output was therefore determined during the third minute of exercise. However, observations made during prolonged severe upright exercise indicate the difficulty in achieving a steady state under these conditions (Figure 8). Thus, in Y.W. the heart rate and cardiac output continued to rise gradually throughout exercise, while in R.M. the heart rate remained relatively steady. It is difficult for untrained subjects to maintain precisely the same work level during prolonged severe exercise, and a constant treadmill speed is no guarantee that the subject's cardiac output will become steady.

The indicator-dilution technic, when combined with a continuous recording of the changes in dye concentration in arterial blood, is well suited for the determination of cardiac output in man during exercise. It is unnecessary to have a catheter in the pulmonary artery to draw mixed venous blood samples, and a steady state needs to be maintained only during the short time required for inscription of the dilution curve. With higher cardiac outputs, the time required for inscription of the indicator-dilution curve becomes less and with the severest exercise in the present experiments the curve was completed within 20 seconds after the injection of dye.

We believe that, when particular attention is paid to obtaining satisfactory calibration curves for the concentration of dye in blood, and when the dye is injected into the central circulation, the indicator-dilution method accurately measures the cardiac output in resting subjects. However, it is probable that the results are less accurate when cardiac output is greatly increased. Under this

circumstance, the area subtended by the dilution curve is much less than that during rest, and hence inaccuracies in its measurement are more likely. Further, as a result of the profound alterations in the systemic distribution of blood flow that occur during exercise, clearance of dye from blood at the sampling site between the end of its primary circulation and its first recirculation in some, but not all, subjects is relatively less complete than when they are at rest. Hence, extrapolation of the disappearance slope in an attempt to exclude the measurement of recirculating dye may be subject to more than the usual inaccuracy.

Stroke volume at rest and during exercise in the upright position. The cardiac index in the subjects resting in the supine position averaged 3.5 L. This is similar to values obtained in young adult males of 3.4 (7), 3.3 (8), 3.6 (9), and 3.5 (10) L by the direct Fick method. The stroke index averaged 54 ml. When the subject stood, the stroke index fell to an average value of 32 ml. Extremely light exercise in the upright position, consisting of contraction and relaxation of the calf muscles but insufficient to increase the oxygen consumption by more than 50 per cent, caused the stroke index to approach the value obtained in the supine position. With exercise resulting in a cardiac index of about 5 to 10 L, the stroke index was similar to that at rest in the supine position, though there were slight variations from one experiment to another. With severe exercise and a cardiac index of up to 12 L, the average value for stroke index for the four subjects was 59 ml.

We are aware of only two studies in which cardiac output and heart rates have been measured in subjects at rest, in the standing position and during exercise. Theilen, Gregg and Rotta (11) found an increase in stroke index from 33 ml, while the subject stood at rest, to 51 ml during moderate exercise (cardiac index 8 L) in seven medical students, at sea level. Mitchell, Sproule and Chapman (12) found an increase in stroke volume from 62 ml while standing to 125 ml during severe exercise when the average cardiac output was 23 L. Our results are similar, the stroke index increasing from 32 to 54 and 56 ml with moderate exercise and to 59 ml with severe exercise. With even the mildest exercise the stroke index increases toward the value obtained while the subject is resting in the supine position, to show only a small further increase above this value even with severe exercise.

Stroke volume during exercise in the supine position. Dexter and co-workers (13) found that light leg exercise while the subject was supine increased the average stroke index from 41 to 51 ml in a group of seven healthy adults. Barratt-Boyes and Wood (10) noted an average increase from 49 to 59 ml with comparable leg exercise in 11 adults. Severe exercise, sufficient to increase the cardiac index to 9 L or more, was undertaken by four subjects in the study of Donald and associates (6). They noted an average increase in stroke index from 55 ml during rest to 62 ml while exercising. Thus during leg exercise while in the supine position, there is a slight increase in stroke volume. The observation in the present study, that the stroke index increased in four subjects by 10 to 20 per cent during severe exercise, supports this conclusion.

Relation of present findings to those of previous workers. Rushmer (1) has discussed the discrepancies in the findings of earlier workers concerning changes in stroke volume during exercise. In his opinion, "the traditional view that stroke volume and heart rate should increase by relatively similar degrees to achieve greater cardiac output stemmed from indirect measurements involving use of CO_2 or foreign gases such as nitrous oxide, acetylene and ethyl iodide. These methods have been largely abandoned in favor of cardiac catheterization and indicator-dilution technics." However, the magnitude of the increase in stroke volume during severe upright exercise depends on the baseline to which it is related (Table II). If this is the value obtained with the subject at rest in the supine position, the increase is slight (-9 to $+23$ per cent) compared with the large increase in heart rate ($+156$ to $+190$ per cent). If it is the value obtained at rest while standing, the increase is considerable ($+61$ to $+110$ per cent) and comparable with the increase in heart rate ($+77$ to $+122$ per cent).

Nevertheless, there is still a conflict between results obtained by the earlier workers who studied changes in stroke volume in subjects sitting on a bicycle ergometer and exercising. Thus, Douglas and Haldane (14), using the carbon dioxide method, found that the stroke volume was

the same with the subject supine, sitting on the cycle, or exercising, while Bock and associates (15), also using the carbon dioxide method, found increases of 36 to 110 per cent with exercise in three normal subjects. Henderson, Haggard and Dolley (16), using the ethyl iodide method, found no increase in stroke volume in untrained subjects, but Christensen (17), using the acetylene method, found that it increased from 60 ml during rest to between 100 and 200 ml during exercise. These discrepancies are not necessarily due to inadequacy of technics. The careful study of Asmussen and Nielsen (18), in which stroke volume was measured simultaneously by the indicator-dilution and acetylene methods, with identical results, suggests that the inert gas methods cannot necessarily be regarded as unsuitable for determination of cardiac output. An alternative explanation might be that the posture and degree of relaxation of the subject sitting on the cycle varied. Thus the resting value for stroke volume of 60 ml obtained by Christensen (17) is similar to that of subjects standing in a relaxed manner, while the resting value obtained by Douglas and Haldane (14) was twice as great, suggesting that their subjects may have been sitting in a less relaxed posture.

The present studies suggest that for a given person the stroke volume increases only slightly once exercise has begun, regardless of whether the subject is supine or standing. During rest, however, the value for stroke volume depends on the body position. The results of earlier workers, such as Bock and associates (15), who used the carbon dioxide method and Christensen (17), who used the acetylene method, are consistent with this interpretation.

SUMMARY

Cardiac output, heart rate, and stroke volume were measured in four healthy, untrained men at rest in the supine position, at rest standing and during exercise in the upright position. The exercise varied in severity from gentle movements of the calf muscles and marking time to walking at 4.5 mph up a treadmill inclined at 12 degrees from the horizontal. This severe exercise was associated with cardiac outputs of 15 to 25 L per minute and oxygen consumptions of 2.0 to 2.8 L per minute.

The cardiac index of subjects at rest in the supine position averaged 3.5 L, and the stroke index averaged 54 ml. When the subject stood, there was a fall in cardiac output and an increase in heart rate. The stroke index averaged 32 ml, a fall of 41 per cent. Mild exercise was sufficient to restore the stroke index almost to the value obtained with the subject at rest in the supine position. With moderate exercise in the upright position the stroke index was similar to that obtained when the subject was at rest in the supine position and with the severest exercise it increased to 59 ml.

It is probable that the apparent discrepancies in previous reports were due to variations in the circumstances under which resting values for stroke volume were obtained rather than to inadequate technics for measuring cardiac output. Thus, if the stroke volume obtained during severe exercise in the upright position is compared with that obtained when the subject is at rest in the supine position, the increase is slight (-9 to $+23$ per cent) compared with the increase in heart rate ($+156$ to $+190$ per cent). If the comparison is made with the value obtained when the subject is standing at rest, the increase in stroke volume ($+61$ to $+110$ per cent) is comparable with that in the heart rate ($+77$ to $+122$ per cent).

ACKNOWLEDGMENT

We wish to thank Miss Lucille Cronin, Mrs. Jean Frank and Miss Rita Schmelzer for their advice and assistance.

REFERENCES

1. Rushmer, R. F. Constancy of stroke volume in ventricular responses to exertion. Amer. J. Physiol. 1959, **196**, 745.
2. Rushmer, R. F., and Smith, O. A., Jr. Cardiac control. Physiol. Rev. 1959, **39**, 41.
3. Wang, Yang, Shepherd, J. T., and Marshall, R. J. Relative contribution of heart rate and stroke volume to changes in cardiac output in man (abstract). Circulation 1959, **20**, 781.
4. Rushmer, R. F. Postural effects on the baselines of ventricular performance. Circulation 1959, **20**, 897.
5. Dubois, D., and Dubois, E. F. The measurement of the surface area of man. Arch. intern. Med. 1915, **15**, 868.

6. Donald, K. W., Bishop, J. M., Cumming, G., and Wade, O. L. The effect of exercise on the cardiac output and circulatory dynamics of normal subjects. Clin. Sci. 1955, **14**, 37.
7. Cournand, A., Riley, R. L., Bradley, S. E., Breed, E. S., Noble, R. P., Lauson, H. D., Gregersen, M. I., and Richards, D. W. Studies of the circulation in clinical shock. Surgery 1943, **13**, 964.
8. Stead, E. A., Jr., Warren, J. V., Merrill, A. J., and Brannon, E. S. The cardiac output in male subjects as measured by the technique of right atrial catheterization. Normal values with observations on the effect of anxiety and tilting. J. clin. Invest. 1945, **24**, 326.
9. Ebert, R. V., Borden, C. W., Wells, H. S., and Wilson, R. H. Studies of the pulmonary circulation. I. The circulation time from the pulmonary artery to the femoral artery and the quantity of blood in the lungs in normal individuals. J. clin. Invest. 1949, **28**, 1134.
10. Barratt-Boyes, B. G., and Wood, E. H. Hemodynamic response of healthy subjects to exercise in the supine position while breathing oxygen. J. appl. Physiol. 1957, **11**, 129.
11. Theilen, E. O., Gregg, D. E., and Rotta, A. Exercise and cardiac work response at high altitude. Circulation 1955, **12**, 383.
12. Mitchell, J. H., Sproule, B. J., and Chapman, C. B. The physiological meaning of the maximal oxygen intake test. J. clin. Invest. 1958, **37**, 538.
13. Dexter, L., Whittenberger, J. L., Haynes, F. W., Goodale, W. T., Gorlin, R., and Sawyer, C. G. Effect of exercise on circulatory dynamics of normal individuals. J. appl. Physiol. 1951, **3**, 439.
14. Douglas, C. G., and Haldane, J. S. The regulation of the general circulation rate in man. J. Physiol. (Lond.) 1922, **56**, 69.
15. Bock, A. V., Vancaulaert, C., Dill, D. B., Fölling, A., and Hurxthal, L. M. Studies in muscular activity. III. Dynamical changes occurring in man at work. J. Physiol. (Lond.) 1928, **66**, 136.
16. Henderson, Y., Haggard, H. W. and Dolley, F. S. The efficiency of the heart and the significance of rapid and slow pulse rates. Amer. J. Physiol. 1927, **82**, 512.
17. Christensen, E. H. Beiträge zur Physiologie schwerer körperlicher Arbeit. V. Mitteilung: Minutenvolumen und Schlagvolumen des Herzens während schwerer körperlicher Arbeit. Arbeitsphysiologie 1931, **4**, 470.
18. Asmussen, E., and Nielsen, M. The cardiac output in rest and work determined simultaneously by the acetylene and the dye injection methods. Acta physiol. scand. 1953, **27**, 217.

ERRATUM

Shafrir, E., and Steinberg, E. The Essential Role of the Adrenal Cortex in the Response of Plasma Free Fatty Acids, Cholesterol, and Phospholipids to Epinephrine Injection. J. clin. Invest. 1960, **39** (February). The authors report an important error in the Methods section. On p. 310, column 2, line 16, read: "150 ml acetic anhydride" instead of "50 ml."

RENAL CONCENTRATING MECHANISMS IN NEWBORN INFANTS. EFFECT OF DIETARY PROTEIN AND WATER CONTENT, ROLE OF UREA, AND RESPONSIVENESS TO ANTI-DIURETIC HORMONE *

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Measurements of the concentration of osmotically active solutes in the urine of dehydrated infants have demonstrated a gradual increase from birth through the first weeks of life, with adult levels being attained only after several months (1–13).

The minimal response noted in infants to administration of vasopressin has led many observers (2, 5, 8, 11, 12) to the conclusion that due to immaturity the renal tubules are relatively insensitive to antidiuretic hormone (ADH). However, failure to observe a decrease in rate of urine flow or an increase in urine osmolality in response to vasopressin cannot be interpreted simply as resistance to the hormone per se, since other aspects of the concentrating mechanism, quite distinct from the role of ADH (14–16), must also be taken into consideration. These include the concentration of sodium in renal tissue along an osmotic gradient from cortex to medulla by active sodium transport in Henle's loop, and maintenance of this gradient, despite the medullary blood flow, through the orientation of the postglomerular capillaries which perform as countercurrent exchangers.

The length of the loop of Henle in its function as a countercurrent multiplier is of considerable importance. The length of this segment in various species correlates well with maximal concentrating performance. It is short in early infancy and increases in length with advancing age (17).

The special role of urea in the concentrating process has been emphasized by Berliner, Levinsky, Davidson and Eden (14) and by Schmidt-Nielsen and O'Dell (18, 19). Protein or urea added to the diet of adults has been shown by Epstein, Kleeman, Pursel and Hendrikx (20) to produce a significant increase in maximal urine osmolality at low rates of urine flow during hydropenia and in the maximal rate of reabsorption of free water ($Tm^{c}_{H_2O}$) during mannitol diuresis. In rats fed urea, Crawford, Doyle and Probst (21) have demonstrated production of smaller daily urine volumes and higher concentrations of both urea and nonurea solutes than in rats treated similarly but not fed urea.

In observations in adults, Epstein and associates (22) and De Wardener and Herxheimer (23) have shown that the response to dehydration and vasopressin administration, as measured by maximal urine osmolality, decreases markedly following several days of forced drinking.

In the present investigation the effects of various levels of intake of protein, urea, sodium chloride, and water on the concentrating mechanism of young full-term and prematurely born infants were examined.

METHODS

Observations were made on full-term and prematurely born infants whose ages and weights are indicated in Table I.

Dietary loading. Paired observations, separated generally by 5 to 10 days, were made on each infant. For 3 to 7 days prior to each observation the infants were maintained on isocaloric diets, as specified in Table I.

In Group I, the diet was varied to provide a high protein intake in a low volume prior to the first observation, and a low protein intake in a high volume prior to the second. The diet of Group 2 was of constant volume but provided a high intake of protein prior to the first observation and a low intake prior to the second. Two infants (Group 2A) were observed while on uniform diets except for the addition of a daily supplement of urea or sodium chloride prior to the first observation.

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TABLE I

Concentrating performance at low and high rates of urine flow

Subject	Birth wt.	Age	Weight	Daily dietary intake: Protein	Daily dietary intake: Volume	Daily dietary intake: Supplementary urea or NaCl	Observations during unmodified hydropenia: Serum P_{osm}*	Serum: Urea N	Urine: V	Urine: U_{osm} Total	Urine: U_{osm} Urea	Observations during i.v. infusion: C_{In}	C_{PAH}	$Tm^{e}_{H_2O}$
	g	*days*	*g*	*g/kg*	*ml/kg*		*mOsm/kg*	*mg/100 ml*	*ml/min/1.73 m²*	*mOsm/kg*		*ml/min/1.73 m²*		
Group 1														
Gi ♀	1,740	29	2,100	8.3	106		318	17.5	0.540	940	303	60.4	194	4.4
		38	2,310	2.5	241		315	13.0	1.13	425	15	51.4	193	0.0
Be ♂	1,640	13	1,740	8.6	114		308	23.0	0.297	857	326			
		20	1,980	2.4	209		313	6.0	0.396	508	17			
As ♀	2,800	25	2,880	9.2	121		310	45.0	0.533	993	214	47.5		4.2
		32	3,170	2.2	213		350	9.0	0.516	690	56	60.4	177	2.0
St ♀	2,800	21	2,900	8.9	117		360	30.0	0.688	933	197	49.9	223	5.2
		28	3,200	2.2	205		319	8.5	0.373	661	71	53.2	248	3.3
Group 2														
He ♀	3,100	7	2,900	8.1	161		340	17.8	0.444	834	298	58.4	161	4.2
		14	2,960	2.2	150		302	9.8	0.250	622	97	59.2	117	2.8
Wh ♂	1,980	11	2,000	9.0	178		312	35.0	0.411	707	336	41.0	93	1.9
		21	2,160	2.4	164		310	8.0	0.197	560	71	33.9	104	1.2
Ca ♂	1,980	11	1,840	9.0	180		308	30.0		712	336			
		22	2,090	2.5	175		295	7.0	0.193	580	54			
Or ♂	3,000	16	2,980	9.1	181		318	19.5	0.564	827	338	44.1	202	
		23	3,180	2.4	167		308	10.0	0.748	690	120	46.5	189	
		25	3,960	8.0	159		325	30.0	0.534	1,139	484	56.6	174	4.4
Mo ♂	3,620	32	4,260	2.6	165		300	12.0	0.380	834	158			
		39	4,440	2.5	165							57.9	203	3.6
Group 2A														
Ri ♀	2,060	9	1,840	2.6	180	Urea, 2.21 g/kg	325	34.5	0.327	825	425	31.3	82	
		14	1,960	2.6	180	None	323	10.0	0.166	730	40	27.5	108	
Pe ♀	2,260	9	2,220	2.3	160	NaCl, 250 mg/kg	312	11.0	0.473	616	69			
		12	2,180	2.3	160	None	330	9.4	0.293	931	124			
Group 3														
Ha ♂	2,140	12	1,880	4.0	117		300	19.3	0.113	615	86			
		19	2,000	3.9	200		310	17.0	0.184	573	183			
Wr ♀	2,560	6	2,440	4.2	132		321	19.2	0.314	825	338	52.2	130	4.6
		13	2,540	3.8	226		290	12.0	0.357	745	223	53.8	197	2.7
Ma ♂	3,240	20	3,510	4.4	127		300	19.0	0.492	864	330			
		27	3,650	4.2	237		312	20.0	0.564	808	376			
Sc ♂	1,960	13	1,970	4.3	124		320	17.0	0.290	657	178	20.9	71	1.6
		20	1,970	3.8	215		297	13.8	0.476	527	191	28.3	88	1.0
Bo ♀	1,900	24	2,240	7.5	123		319	32.0	0.293	670	263	53.7	197	3.4
		17	1,980	7.5	214		340	34.0	0.355	595	296	54.0	186	2.3

* Several values for P_{osm} are unexpectedly high. Although these values generally were associated with elevated serum electrolyte concentrations, later observations following the same degree of water deprivation failed to yield similarly elevated values, suggesting a methodological error. However, assuming such error, only slight and insignificant differences would result in derived data and therefore the figures for serum osmolality were used as shown.

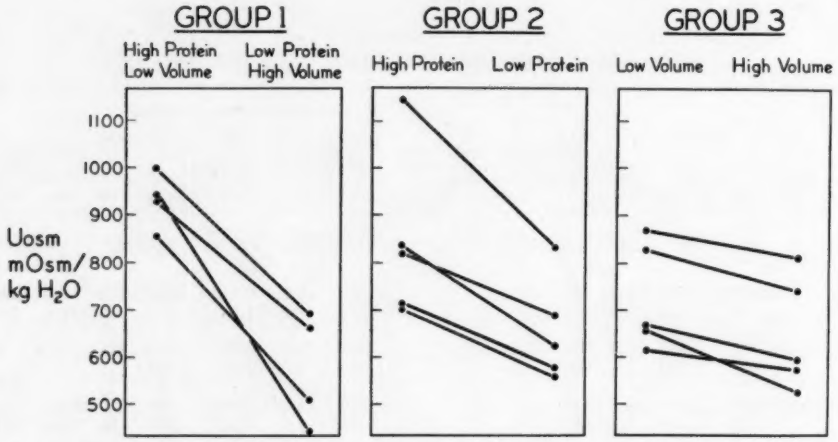

FIG. 1. EFFECT OF DIETARY PROTEIN AND WATER CONTENT ON MAXIMAL URINARY OSMOLALITY MEASURED DURING HYDROPENIA.

The diet of Group 3 was constant except for water content, providing a low volume prior to the first and high volume prior to the second observation.

The observations were arranged so that any increase in concentrating performance that might result from greater maturity with increasing age would be opposite to the effect expected from the particular diet. The infants were thirsted and fasted uniformly for 12 to 14 hours prior to each observation.

For the determination of maximal concentrating performance at low rates of urine flow, several timed urine specimens were obtained at the beginning of each observation, the infants being disturbed as little as possible. In order to obviate the introduction of a painful stimulus, which has been shown in infants to influence both glomerular filtration rate (GFR) and ADH release, vasopressin was not administered during the initial observation periods. However, it has been demonstrated repeatedly that vasopressin does not augment the maximal ADH effect resulting from dehydration. Following the initial collection periods, a majority of subjects was given a saline infusion and priming injection containing inulin, para-aminohippurate (PAH), and vasopressin. The priming dose of vasopressin was 50 mU per m^2, the sustaining dose 0.75 mU per minute per m^2. All fluids were infused at a constant rate of 10 to 12 ml per minute per 1.73 m^2. After equilibration and 2 to 4 additional collection periods, mannitol was added to the infusion in a concentration of 10 per cent and collections were continued until the rate of urine flow reached a plateau.

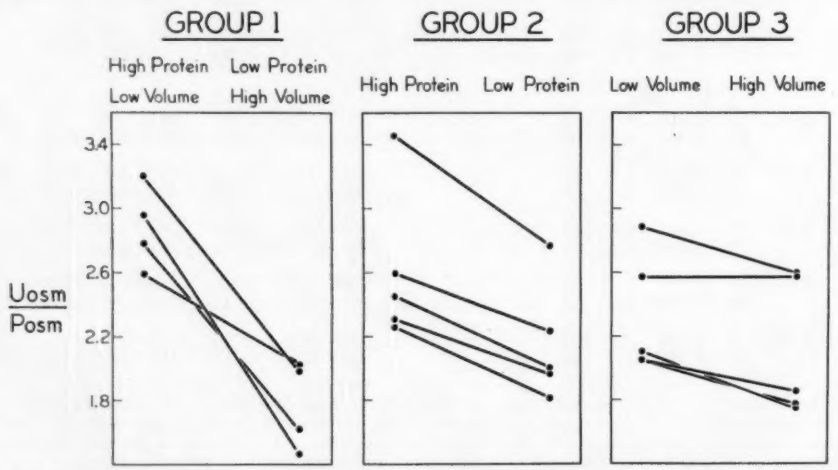

FIG. 2. EFFECT OF DIETARY PROTEIN AND WATER CONTENT ON MAXIMAL U/P OSMOLAL RATIO MEASURED DURING HYDROPENIA.

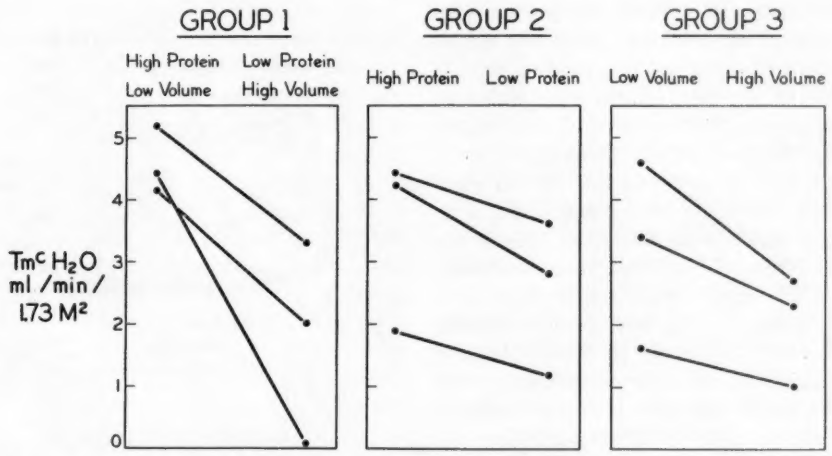

FIG. 3. EFFECT OF DIETARY PROTEIN AND WATER CONTENT ON $Tm^c_{H_2O}$ MEASURED DURING MANNITOL DIURESIS AND VASOPRESSIN ADMINISTRATION.

Acute loading. Several unpaired observations were carried out on infants maintained on a diet providing 2.3 to 2.6 g of protein per kg per day and 160 to 180 ml per kg per day. These observations likewise were preceded by a period of 12 to 14 hours of complete thirsting and fasting. After several hours of baseline observation, urea was administered through a stomach tube and additional urine samples were collected for 2 to 3 hours.

The osmolality of urine and serum was determined by measurement of the freezing point depression, using a Fiske osmometer with a semi-micro adapter. Urea nitrogen was determined by a modification of the methods of Friedman (24), Marsh, Fingerhut and Kirsch (25), and Natelson (26); inulin was determined by a modification of the method of Schreiner (27); and PAH by a modification of the method of Smith and co-workers (28).

RESULTS

Group 1: High protein, low volume diets and low protein, high volume diets (Figures 1, 2 and 3, Tables I-1 and II-1). Following water deprivation, significantly greater values for maximal urine osmolality, maximal urine-plasma (U/P) osmolal ratio, and $Tm^c_{H_2O}$ were observed in each infant following ingestion of the high protein, low volume

TABLE II

Statistical analysis of derived data

Group	Diet	Serum urea nitrogen	V	U_{osm}		$U_{osm}V$		C_{osm}	$T^c_{H_2O}$	$Tm^c_{H_2O}$
		mg/100 ml	*ml/min 1.73 m²*	Total	Urea	Total	Urea			
				mOsm/kg		*μOsm/min/1.73 m²*		*ml/min/1.73 m²*		
1	High protein, low volume	28.9	0.514	931	260	485	128	1.48	0.96	4.60
	Low protein, high volume	9.1	0.604	571	40	321	19	0.99	0.38	1.77
	p*	0.05	0.7	<0.01	0.02	0.1–0.2	<0.01	0.1	0.01	0.05–0.1
2	High protein	26.5	0.488	844	358	434	180	1.34	0.85	3.50
	Low protein	9.4	0.394	657	100	275	47	0.90	0.51	2.52
	p	0.01	0.4	<0.01	<0.001	0.10	0.01	0.1–0.2	0.05–0.1	0.05
3	Low volume	21.3	0.300	726	239	228	81	0.73	0.43	3.2
	High volume	21.4	0.387	650	254	258	104	0.84	0.45	2.0
	p	1.0	0.02–0.05	<0.01	0.7	0.02–0.05	0.10–0.20	0.05–0.1	0.5	0.05–0.1

* Student's *t* test for paired data (29).

diets. The concentration of blood urea nitrogen and the rates of excretion of osmotically active solutes were greater during observations following ingestion of the high protein diets, although the differences observed in rates of total solute excretion are of questionable significance.

Group 2: High protein and low protein diets (Figures 1, 2 and 3, Tables I-2 and II-2). Results in these infants were similar to those in the first group. Values for maximal urine osmolality, maximal U/P osmolal ratio, and $Tm^{c}_{H_2O}$ were consistently greater during hydropenia in infants given high protein feeding. In one 25 day old infant, the concentration of osmotically active urinary solutes was as high as 1,139 mOsm per kg. The decrease in maximal urine osmolality observed after low protein feeding paralleled the decrease in urine urea content.

Group 2A: Effect of adding urea or sodium chloride to the diet (Table I-2A). In one premature infant, the addition of urea to the diet, in an amount calculated to provide a rate of urea excretion comparable to that observed during high protein feeding, resulted in changes in concentrating performance similar to those observed following diets of high protein. As in the first and second group of infants, the rate of excretion of urea, the blood urea nitrogen concentration, and the urine urea nitrogen concentration were greater following ingestion of the diet with urea supplement.

The addition of sodium chloride to a basal diet in place of urea as a solute load was studied in one infant. Rates of excretion of total and nonurea solute were greater following sodium chloride supplementation, although the increases were slight and not as great as those after urea supplementation. However, maximal urine osmolality and U/P osmolal ratio were lower following ingestion of the diet with added salt.

Effect of acute urea loading (Figure 4). Administration of urea, in amounts ranging from 50 to 300 mg per kg body weight, resulted in three types of response; representative observations are diagrammed in Figure 4. Following small urea loads, no change was noted in urine volume or osmolality. In the majority of infants, striking and proportional increases in both rates of urine flow and rates of solute excretion were observed, but with no changes in the concentration of total urinary solute. Nevertheless, these infants did demonstrate an increase in the ratio of urea to nonurea solute. In several infants, increases in both rates of urine flow and of solute excretion were noted, accompanied by an increase in concentration of total urinary solutes. Increases in rates of solute excretion with urea loading were found to be the result primarily of increases in rates of urea excretion, since the increase in urine osmolality was almost exclusively in the urea fraction.

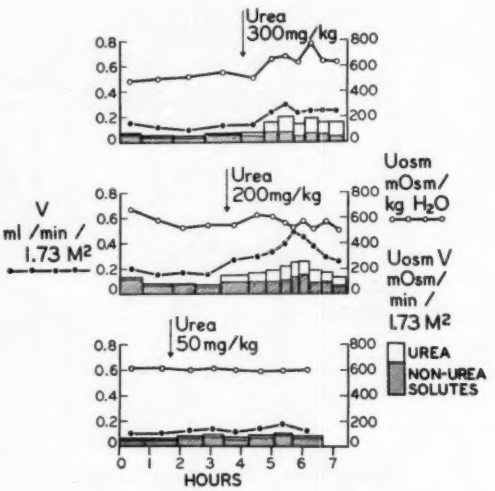

FIG. 4. EFFECT OF ACUTE UREA LOADING ON RATE OF URINE FLOW, RATES OF EXCRETION OF TOTAL, UREA, AND NONUREA SOLUTE, AND CONCENTRATION OF URINARY OSMOTICALLY ACTIVE SOLUTE.

Group 3: Low volume and high volume diets (Figures 1, 2 and 3, Tables I-3 and II-3). Values observed for maximal urine osmolality, maximal U/P osmolal ratio, and $Tm^{c}_{H_2O}$ were greater following feeding of low volume diets. In contrast with the other groups, these findings were accompanied by significantly lower rates of urine flow. The greater concentrations of osmotically active solutes in the urine were accompanied by decreased rates of total solute excretion and osmolal clearance, with no change in the rates of excretion of urea.

DISCUSSION

Current evidence suggests that the action of the antidiuretic hormone is to increase the permeability of certain segments of the renal tubule to

water and that urine hypertonic to plasma is produced by the abstraction of water from an isotonic precursor (14). During hydropenia, with minimal rates of urine flow, only very small volumes of osmotically free water must be reabsorbed at the concentrating site to produce urine several times hypertonic to plasma. Theoretically, reabsorption in the collecting tubule of only 2 ml of water per minute per 1.73 m^2, concomitant with a rate of urine flow of 0.5 ml per minute per 1.73 m^2, would permit formation of urine with a solute concentration of 1,500 mOsm per kg. Measurements in infants of the rate of reabsorption of osmotically free water during mannitol diuresis (Table I) yield values as high as 3 to 4 ml per minute per 1.73 m^2, indicating that in infants, as in adults, the factor limiting maximal urinary osmolality at low rates of urine flow is not the maximal rate at which tubular water can be reabsorbed. Thus it would appear that the failure of the young infant to achieve levels of urinary osmolality observed in the adult during prolonged dehydration is not due to inadequate permeability of the tubule to water in response to ADH. This suggests the possibility that differences may exist between infants and adults in the mechanisms responsible for the production of an area of hypertonicity around the collecting tubule.

The usual dietary intake of protein in infants is relatively high when compared with adults on the basis of body weight but is approximately the same when compared on the basis of surface area. It is not possible to establish a physiological standard of reference for such comparisons (30). However, independent of standards of reference, the infant excretes a considerably smaller percentage of the nitrogen of his diet than does the adult, due to a strongly anabolic state. In addition, in the very young infant the percentage of total nitrogen excreted as urea is lower than in the adult, due mainly to excretion of a larger proportion of nitrogen in the form of amino acids and ammonia (31, 32).

In the present observations, infants fed high protein diets demonstrated a consistently increased concentrating performance following water deprivation when compared with infants fed low protein diets. These findings in infants are in agreement with those reported in adults by Epstein and co-workers (20). In Group 2, values for P_{osm} were consistently greater following high protein intake, suggesting that the higher values for urinary osmolality observed may have resulted in part from a greater degree of dehydration or ADH release. As discussed earlier, however, it is believed that the experimental conditions resulted in all instances in maximal ADH release; analysis of body weight loss following dehydration indicated no differences with the various dietary regimens. Measurement of the osmotically active constituents of the urine, separated into urea and nonurea solute, showed that the decrease in urine osmolality from the first to the second observation was due primarily to a decrease in concentration of urea, resulting from a decrease in the rate of urea excretion, with no major change in concentration of nonurea solutes. The decrease in rate of urea excretion was due principally to the decreased concentration of blood urea nitrogen, rather than to changes in GFR or in rates of urea clearance, although decreases in the latter associated with lower rates of urine flow were observed in the second group of infants with low protein feeding.

Osmolal clearances were greater during the observations preceded by high protein feeding. Despite this presumably greater volume of isotonic fluid delivered to the concentrating site and the increased solute load, urine osmolality and urine U/P osmolal ratio were increased, suggesting (33) an enhanced rate of reabsorption of water in the collecting tubule ($T^c_{H_2O}$). Addition of urea to the diet, in place of protein, resulted in similar findings, indicating further that the increased concentrating performance observed following high protein feeding most likely resulted simply from the provision of additional urea for excretion.

When sodium chloride was added to the diet for several days as an osmotic load in place of increased protein or urea, the osmolal clearance was greater than that on the same diet without added salt. However, the maximal urinary osmolality and the U/P osmolal ratio in response to dehydration were decreased.

Thus the unique action of urea as a urinary solute, originally suggested by Gamble and associates (34, 35) and recently confirmed by Crawford and co-workers (21) and others (19, 20, 36–38) can be demonstrated in infants.

Epstein and co-workers (20) found in adults that acute urea loading had no effect on maximal urine osmolality or $Tm^{c}_{H_2O}$ and concluded that the effect of chronic administration of protein or urea was achieved by promoting an adaptive response by the renal tubules. In the present study similar results were obtained in infants given low or intermediate urea loads. However, these data are interpreted as showing that the effect of urea can be demonstrated not only after chronic administration but also with acute loading, since it would be expected *a priori* that the osmotic diuresis resulting from acute urea loading would be accompanied by a decrease in urinary osmolality (33), which did not occur. The demonstration in several infants of unchanged urine osmolality following urea loading, despite increased rates of solute excretion and urine flow, indicates an enhanced rate of reabsorption of water in the concentrating site. Furthermore, in three subjects an increase in urine osmolality following urea loading was observed, as recently reported in adults by Levinsky and Berliner (39), adding to the evidence supporting the unique role of urea.

The feeding schedule of the young infant, providing nourishment every 3 to 4 hours during the newborn period and allowing neither fasting nor thirsting for a period of more than 6 to 8 hours during the remainder of early infancy, contrasts markedly with the feeding schedule of the adult, in which complete thirsting and fasting commonly occur for 12 or more hours each day. The daily intake of fluid and the rate of turnover of body water is large in the infant when compared with the adult. In the present investigation the importance of fluid intake on the urinary concentrating performance in infants was demonstrated. Lower rates of urine flow and higher values for urinary osmolality were observed during dehydration following diets of low volume, similar to the findings previously reported in adults (22, 23).

Following ingestion of low volume diets, rates of osmolal clearance and of excretion of total osmotically active solutes, measured during hydropenia, were less than those after ingestion of high volume diets. Values for $T^{c}_{H_2O}$ were not different in infants previously fed low or high volume diets, suggesting that the smaller urine volumes noted after low volume diets might represent the net effect of reabsorption of a constant volume of osmotically free water from a smaller volume of isotonic precursor fluid in the collecting tubule.

SUMMARY AND CONCLUSIONS

1. Observations were made of the effects of various levels of intake of protein, urea, sodium chloride, and water on the concentrating mechanism of young full-term and prematurely born infants.

2. In terms of current concepts of renal concentrating mechanisms, the failure of the young infant consistently to produce urine as hypertonic to plasma as that seen in the adult appears to be explained best, not by differences in tubular permeability to water in response to antidiuretic hormone, but rather through differences in diet and metabolism of protein, and most likely through differences in other mechanisms involved in the production of an area of interstitial hypertonicity around the collecting tubule.

3. The young infant normally receives a diet high in protein content. However, by virtue of his strongly anabolic state, as well as the form utilized for urinary nitrogen, little urea is available for excretion. High protein intake, or supplementation of the diet with urea, provides a significantly increased amount of urea for excretion, and results during hydropenia in a greatly increased urinary osmolality and urine urea concentration.

4. The usual infant diet provides a large intake of water. Ingestion of a diet with low water content results in increased urinary osmolality in response to dehydration.

5. Values for net reabsorption of osmotically free water in infants approach those seen in adults, indicating no difference in the effect of antidiuretic hormone on the permeability of the collecting tubule to water.

REFERENCES

1. McCance, R. A., and Young, W. F. The secretion of urine by newborn infants. J. Physiol. (Lond.) 1941, **99**, 265.
2. Heller, H. The renal function of newborn infants. J. Physiol. (Lond.) 1944, **102**, 429.
3. Thomson, J. Observations on the urine of the newborn infant. Arch. Dis. Childh. 1944, **19**, 169.
4. Thomson, J. Urinalysis in dehydration fever. Arch. Dis. Childh. 1947, **22**, 226.

5. Barnett, H. L., Hare, K., McNamara, H., and Hare, R. Measurement of glomerular filtration rate in premature infants. J. clin. Invest. 1948, **27**, 691.
6. Pratt, E. L., Bienvenu, B., and Whyte, M. M. Concentration of urine solutes by young infants. Pediatrics 1948, **1**, 181.
7. McCance, R. A. Renal physiology in infancy. Amer. J. Med. 1950, **9**, 229.
8. Heller, H. The water metabolism of newborn infants and animals. Arch. Dis. Childh. 1951, **26**, 195.
9. Barnett, H. L., and Vesterdal, J. The physiologic and clinical significance of immaturity of kidney function in young infants. J. Pediat. 1953, **42**, 98.
10. Hansen, J. D. L., and Smith, C. A. Effects of withholding fluid in the immediate postnatal period. Pediatrics 1953, **12**, 99.
11. Ames, R. G. Urinary water excretion and neurohypophysial function in full term and premature infants shortly after birth. Pediatrics 1953, **12**, 272.
12. Calcagno, P. L., Rubin, M. I., and Weintraub, D. H. Studies on the renal concentrating and diluting mechanisms in the premature infant. J. clin. Invest. 1954, **33**, 91.
13. McCance, R. A., and Widdowson, E. M. Normal renal function in the first two days of life. Arch. Dis. Childh. 1954, **29**, 488.
14. Berliner, R. W., Levinsky, N. G., Davidson, D. G., and Eden, M. Dilution and concentration of the urine and the action of antidiuretic hormone. Amer. J. Med. 1958, **24**, 730.
15. Thorn, N. A. Mammalian antidiuretic hormone. Physiol. Rev. 1958, **38**, 169.
16. Lamdin, E. Mechanisms of urinary concentration and dilution. A. M. A. Arch. intern. Med. 1959, **103**, 644.
17. Peter, K. Studies of the Growth and Development of the Kidney. Jena, Gustav Fischer, 1927.
18. Schmidt-Nielsen, B. Urea excretion in mammals. Physiol. Rev. 1958, **38**, 139.
19. Schmidt-Nielsen, B., and O'Dell, R. Effect of diet on distribution of urea and electrolytes in kidneys of sheep. Amer. J. Physiol. 1959, **197**, 856.
20. Epstein, F. H., Kleeman, C. R., Pursel, S., and Hendrikx, A. The effect of feeding protein and urea on the renal concentrating process. J. clin. Invest. 1957, **36**, 635.
21. Crawford, J. D., Doyle, A. P., and Probst, J. H. Service of urea in renal water conservation. Amer. J. Physiol. 1959, **196**, 545.
22. Epstein, F. H., Kleeman, C. R., and Hendrikx, A. The influence of bodily hydration on the renal concentrating process. J. clin. Invest. 1957, **36**, 629.
23. De Wardener, H. E., and Herxheimer, A. The effect of a high water intake on the kidney's ability to concentrate the urine in man. J. Physiol. (Lond.) 1957, **139**, 42.
24. Friedman, H. S. Modification of the determination of urea by the diacetyl monoxime method. Analyt. Chem. 1953, **25**, 662.
25. Marsh, W. H., Fingerhut, B., and Kirsch, E. Determination of urea nitrogen with the diacetyl method and an automatic dialyzing apparatus. Amer. J. clin. Path. 1957, **28**, 681.
26. Natelson, S. Microtechniques of Clinical Chemistry for the Routine Laboratory. Springfield, Ill. Charles C Thomas, 1957, p. 381.
27. Schreiner, G. E. Determination of inulin by means of resorcinol. Proc. Soc. exp. Biol. (N. Y.) 1950, **74**, 117.
28. Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M. The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. J. clin. Invest. 1945, **24**, 388.
29. Mainland, D. Elementary Medical Statistics; The Principles of Quantitative Medicine. Philadelphia, W. B. Saunders Company, 1952, p. 149.
30. Edelmann, C. M., Jr., and Barnett, H. L. Role of the kidney in water metabolism in young infants. Physiologic and clinical considerations. J. Pediat. 1960, **56**, 154.
31. Barlow, A., and McCance, R. A. The nitrogen partition in newborn infants' urine. Arch. Dis. Childh. 1949, **24**, 225.
32. Widdowson, E. M., Dickerson, J. W. T., and McCance, R. A. The excretion of urea, ammonia and purine end-products by the newborn animal. Biochem. J. 1958, **69**, 421.
33. Raisz, L. G., and Scheer, R. L. Studies on the renal concentrating mechanism. II. Effect of small acute changes in solute excretion. J. clin. Invest. 1959, **38**, 1.
34. Gamble, J. L., Putnam, M. C., and McKhann, C. F. The optimal water requirement in renal function. 1. Measurements of water drinking by rats according to increments of urea and of several salts in the food. Amer. J. Physiol. 1929, **88**, 571.
35. Gamble, J. L., McKhann, C. F., Butler, A. M., and Tuthill, E. An economy of water in renal function referable to urea. Amer. J. Physiol. 1934, **109**, 139.
36. Kellogg, R. H., and Koike, T. I. Difference between mannitol and urea diuresis in the rat (abstract). Amer. J. Physiol. 1955, **183**, 633.
37. Rubini, M. E., Blythe, W. B., and Meroney, W. H. Effect of the type of urinary solute on the process of urine concentration (abstract). J. clin. Invest. 1957, **36**, 926.
38. Hendrikx, A., and Epstein, F. H. Effect of feeding protein and urea on renal concentrating ability in the rat. Amer. J. Physiol. 1958, **195**, 539.
39. Levinsky, N. G., and Berliner, R. W. The role of urea in the urine concentrating mechanism. J. clin. Invest. 1959, **38**, 741.

AN IMMUNOCHEMICAL ASSAY OF TOTAL EXTRACTABLE INSULIN IN MAN *

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For the precise assay of minute quantities of insulin in biological fluids, techniques using fibril formation (1) or paper chromatography (2), although specific for the insulin molecule, lack the required sensitivity. Methods based on the measurement *in vivo* of a fall in blood sugar in surgically modified rats (3) afford sensitivity, but lack both specificity and precision. *In vitro* biological assays, in which glucose metabolism is measured in rat diaphragm or epididymal fat, while perhaps more precise than *in vivo* methods, are subject to interference by other hormones such as somatotropin and epinephrine (4, 5), by unknown "insulin-like" substances whose extraction characteristics differ from those of insulin (6) and by numerous inhibitors of insulin activity (7–10).

In contrast, an immunological method should be both chemically specific and, especially after application of an isotopic method for the immunologic quantitation, reasonably sensitive and precise.

Berson and co-workers have originally reported (11) that when insulin-I^{131} is added to an excess of antibodies, it rapidly becomes bound. Furthermore, addition of unlabeled insulin in increasing quantities results in gradual saturation of the binding sites, reflected by an increase in the percentage of unbound insulin-I^{131}. We have subsequently confirmed these observations (12). The change in the percentage of unbound labeled insulin forms the basis of methods recently described for the measurement of injected insulin in rabbit plasma (13) and extracted insulin from mouse pancreas (14). Both of these methods utilized hydrodynamic flow techniques for the determination of the percentage of unbound insulin.

The modification of this approach for the assay of circulating human insulin is the subject of this report. Specific consideration has been given the following points: *1*) the preliminary extraction of the serum to remove endogenous antibodies that would interfere in the assay; *2*) the effective separation, by means of preferential salt precipitation, of free insulin-I^{131} from the bound labeled hormone produced after incubation with antibody and the elimination of nonspecific binding of insulin-I^{131} to extraneous proteins in the extracts; *3*) the cross reaction of human insulin with antibodies formed against beef insulin in the guinea pig; and *4*) the preparation of appropriate standard incubates with protein concentration and pH most nearly approximating those of the unknown extracts.

The described method was used to investigate the effect of carbohydrate ingestion, insulin injection and the presence of acromegaly on the levels of circulating insulin in man.

MATERIALS AND METHODS

Bovine crystalline insulin, lot no. T-2842, was obtained from Eli Lilly & Company. Insulin-I^{131} was prepared by a modification of the method of Burrows, Peters and Lowell (15), as described previously (12). *Insulin antisera* were obtained from guinea pigs treated as follows: 1 mg of insulin in 2 ml of Freund's adjuvant (16) was injected subcutaneously into adult guinea pigs. Eight days later the animals were re-injected with 0.5 mg of insulin in the adjuvant. When necessary, animals were given 10 per cent glucose intraperitoneally to overcome severe hypoglycemia. After 5 months the animals were injected with 0.25 mg of insulin in 2.5 ml of 10 per cent glucose. (The 5 month interval was arbitrarily chosen to conform with a concurrent study and does not represent an experimentally determined optimal period.) Anaphylaxis was general, but animals given adrenaline survived. Six days after the final injection blood was drawn by cardiac puncture; serum from the specimens was frozen and stored.

Expressing the insulin antibody titer as the dilution of serum, 1 ml of which would still bind 50 per cent of 0.1 μg of insulin-I^{131}, a typical antiserum had a value of 224 (12). Antiserum was diluted with 5 per cent human albumin until 10 μl bound about 75 per cent of 16 μU of

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TABLE I

*Inactivation of insulin-I^{131} in plasma measured by hydrodynamic flow (12)**

Experiment no	Before treatment			After 24 hours of treatment		
	Origin	Moving fraction	Insulin-degraded	Origin	Moving fraction	Insulin-degraded
			%			%
A. Alkali inactivation						
1	186	34	14	32	178	85
2	125	24	16	15	105	88
3	126	14	9	22	103	82
B. Cysteine inactivation						
1	125	22	18	87	53	38
2	108	14	11	57	30	34

* All values are given as activity in counts per second.

insulin-I^{131} (1 mg insulin considered equivalent to 27 units).

"Insulin-free" plasma, for use as carrier protein, was prepared by adjusting plasma to pH 13.0 with concentrated ammonium hydroxide and storing it at 4° C for 24 hours. Final pH was adjusted to 7.4 with concentrated hydrochloric acid. The effectiveness of alkaline inactivation of insulin was demonstrated by adding 0.1 μg of insulin-I^{131} to 1 ml of plasma, treating it as described above and subjecting the final mixture to hydrodynamic flow (insulin remains at the origin; degradation products move with serum proteins). Approximately 85 per cent of the radioactivity was found in the fraction representing degradation products (Table I). In similar samples to which 0.025 M cysteine (17) had been added, degradation during a 24 hour period at 4° C amounted to only 35 per cent.

Extraction of insulin from serum. To prevent the degradation of insulin that may occur during clot formation at room temperature, particularly when hemolysis has occurred, whole blood was allowed to clot in an ice bath.

A method for extracting insulin from pancreatic tissue (18) was modified for use with the resulting serum as follows. A mixture of 5 ml of serum and 20 ml of acidic alcohol (15 ml of 12 N hydrochloric acid diluted to 1 L with 75 per cent ethanol) was agitated for 1 hour at room temperature. After centrifugation, the supernatant was removed. The precipitate was re-extracted for 1 hour with an additional 20 ml of the acidic alcohol solution and centrifuged. The two supernatants were combined and adjusted to pH 8.5 to 9.0 with concentrated ammonium hydroxide. After 30 minutes at 4° C insoluble proteins were removed by centrifugation. To precipitate the crude insulin, 4 volumes of an alcohol-ether solution (1.5:2.5 vol/vol) were added and the mixture was chilled overnight at 4° C. After centrifugation the precipitate was exposed to a jet of air for 60 seconds to remove excess ether. The still moist precipitate was dissolved in 1.5 ml of 0.2 M glycine buffer (pH 8.5) by vigorous stirring with a glass rod. After a 1 or 2 minute interval to allow foam to settle, the solution was adjusted to 2.5 ml with additional glycine buffer. Protein that failed to go into solution was insulin-free and could be discarded. The final extract, representing a twofold concentration of the initial serum, was sufficient for 5 determinations and could be stored at 4° C for up to 2 weeks.

Assay of insulin. To obtain a standard curve, various amounts of nonradioactive insulin in 10 μl of 5 per cent albumin were incubated for 1 hour at 25° C with a mixture of 0.5 ml of 5 per cent albumin in the 0.2 M glycine buffer, 0.5 ml of 30 per cent urea in 5 per cent albumin solution, 10 μl (16 μU) of insulin-I^{131}, and 10 μl of the diluted guinea pig antiserum. After incubation, 0.4 ml of the "insulin-free" human plasma was added as carrier protein. The insulin bound to antibody was precipitated by adding 2 volumes of water containing sufficient sodium sulfite to make a final concentration of 17 per cent in the incubation mixture. The contents of the tubes were mixed and allowed to stand 1 hour at 4° C.

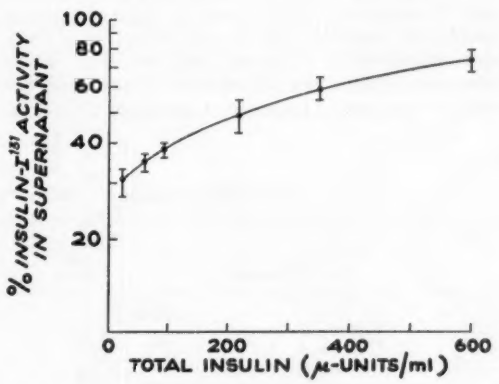

FIG. 1. EFFECT OF CONCENTRATION OF TOTAL INSULIN ON LEVEL OF FREE INSULIN-I^{131} REMAINING IN SUPERNATANT AFTER PRECIPITATION WITH SODIUM SULFITE. Mean of curves determined on 11 days during 2 month period ($\overline{\mid}$ = mean $\pm$ 2 $\times$ SD).

The precipitated proteins were removed by centrifugation, and the radioactivity in an aliquot of the supernatant was counted in a well-type scintillation counter. When corrected for total volume, the value obtained represented the free insulin (and probably some degradation products) remaining after incubation. The standard curve was obtained by plotting the activity in the supernatant as per cent of the total radioactivity initially added against the concentration of total insulin (Figure 1).

Since the percentage of unbound insulin did not increase linearly with, or as a logarithmic function of, insulin concentration, at least 5 points along the curve were required for each day's determination. The curve was quite reproducible; over a period of 2 months during which several different preparations of insulin-I^{131} were used, the standard deviation for the low point and high point was 3.1 and 3.8 per cent, respectively ($n = 11$).

The unknown insulin was assayed by substituting 0.5 ml portions of the serum extract (equivalent to 1 ml of serum) for the glycine-albumin buffer used in the standards. Incubation and salt precipitation were carried out as described above. To determine the insulin content of the unknown sample the percentage of radioactivity in the resulting supernatant was read against the standard curve. Total insulin in serum was calculated by dividing this value by 0.8 (the mean per cent recovery of insulin by the extraction procedure).

Preparation and assay of human insulin. One g aliquots of biopsy specimens of a fresh human pancreas and of an islet cell tumor were extracted by an acid-alcohol procedure (14). The final precipitate from alcohol-ether was dissolved in 1 ml of dilute hydrochloric acid and stored as a stock source of crude human insulin. Prior to immunological assay, the stock extracts were diluted 500-fold with the glycine albumin buffer. The biological activity of these extracts was also determined by the mouse convulsion assay (19). Since only 10 animals were used for each standard or unknown sample, the results of this bioassay served as a crude approximation only. However, past experience in our laboratory indicates that variations in results rarely exceed 100 per cent. In addition, a sample of the extract from normal pancreas was determined more precisely by Dr. Otto Behrens in the laboratories of Eli Lilly & Company.

RESULTS

Extraction of insulin from serum. Table II shows the recoveries obtained when 1,250 μU of insulin-I^{131} was added to 5 ml of serum and extracted one hour later by the acid-alcohol procedure. Since degradation products produced during the iodination or the extraction procedures could have accounted for part of the radioactivity, aliquots of both the starting serum containing the labeled hormone and the final extract were subjected to hydrodynamic flow. The tabulated values have been corrected and represent the portion of the label that was chromatographically similar to insulin. When normal serum was extracted, approximately 80 per cent of the added insulin was recovered. As the donors had never received insulin, the serum did not contain significant amounts of endogenous antibodies (Table II). Recovery of insulin-I^{131} from sera (which had insulin antibody titers of 7 and 16, respectively) of two insulin-resistant diabetics was then evaluated. Despite the almost quantitative binding of the added insulin-I^{131}, recovery after extraction was essentially the same as that from normal serum. When extracts prepared from resistant sera were incubated with insulin-I^{131} and subjected to hydrodynamic flow, insulin-binding was no longer demonstrable. A final recovery of 80 per cent was assumed for all sera subsequently assayed for insulin content. No significant decomposition of the insulin could be demonstrated by hydrodynamic flow in extracts stored for as long as two

TABLE II

Acid-alcohol extraction of insulin-I^{131} from normal and insulin-resistant sera

Source of serum	Antibody titer* of serum	Insulin-I^{131} bound to serum protein	Recovery of insulin-I^{131} after extraction	Antibody titer of final extract
		%	%	
Normal	0	5	78	
Normal	0	11	80	
Normal	0	3	83	
Insulin-resistant diabetic	7	93	84	0
Insulin-resistant diabetic	16	96	80	0

* Titer = the dilution of serum, 1 ml of which will still bind 50 per cent of 0.1 μg of insulin-I^{131}.

weeks at 4° C. According to estimates by both biuret and Kjeldahl nitrogen methods, only 15 to 19 per cent of the total protein remained after the extraction procedure.

Solubility of insulin-I^{131} and insulin-I^{131} antibody in sodium sulfite. Attempts to precipitate preferentially the insulin antibody complex with increasing amounts of trichloroacetic acid proved unsuccessful, since both the free and bound insulins precipitated simultaneously. Preliminary experiments substituting sodium sulfite as a precipitant indicated that this approach was feasible, provided that carrier protein was added. "Insulin-free" plasma or gelatin was utilized as carrier

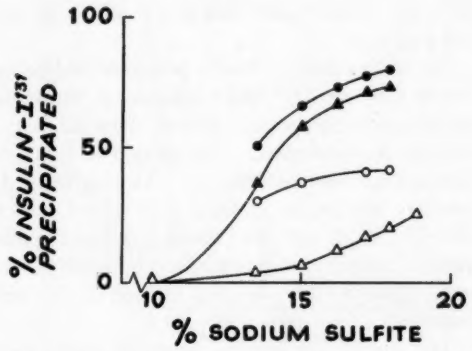

FIG. 2. PREFERENTIAL PRECIPITATION OF FREE AND BOUND INSULIN-I^{131} IN ALBUMIN-GLYCINE BUFFER AND SERUM EXTRACTS BY SODIUM SULFITE. ▲——▲ Bound insulin-I^{131} (antibody added) in buffer; △——△ free insulin-I^{131} (no antibody) in buffer; ●——● bound insulin-I^{131} (antibody added) in serum extract; ○——○ free insulin-I^{131} (no antibody) in serum extract.

protein. The latter was eventually discarded when it proved too soluble in sodium sulfite at the pH of 8.5 used for the assay.

Since the standards contained less precipitable protein than did the unknown extracts, it was necessary to add sufficient carrier protein to both solutions to minimize the difference. Too little carrier caused incomplete precipitation in the standards, resulting in erroneously low values for insulin in the extracts. The concentration of carrier protein necessary to overcome this situation, but not great enough to increase coprecipitation of the free insulin, was determined by adding increasing amounts of carrier to incubates of insulin-I^{131} plus antibody both in the standard glycine-

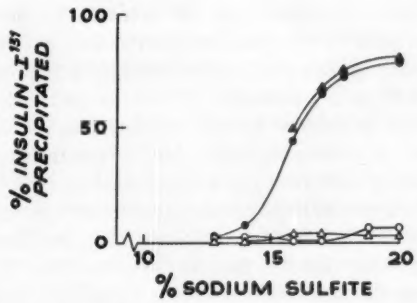

FIG. 3. EFFECT OF 15 PER CENT UREA ON PREFERENTIAL PRECIPITATION OF FREE AND BOUND INSULIN-I^{131} IN ALBUMIN-GLYCINE BUFFER OR SERUM EXTRACTS BY SODIUM SULFITE. ▲——▲ Bound insulin-I^{131} (antibody added) in buffer; △——△ free insulin-I^{131} (no antibody) in buffer; ●——● bound insulin-I^{131} (antibody added) in serum extract; ○——○ free insulin-I^{131} (no antibody) in serum extract.

albumin buffer and in an extract of "insulin-free" plasma until the resulting supernatant counts in each were identical.

Figure 2 shows the effect of the concentration of sodium sulfite on the solubility of insulin-I^{131} suspended in glycine-albumin buffer or serum extract, with and without the addition of sufficient antibody to bind approximately 80 per cent of the labeled hormone. In the glycine-albumin solutions a concentration of 15 per cent sodium sulfite resulted in quantitative precipitation of insulin-I^{131} bound to antibody, whereas free insulin-I^{131} remained in solution. In contrast, when serum extracts were incubated without antibody, 40 per cent of the total insulin-I^{131} was bound and precipitated by extraneous proteins contained in the extract.

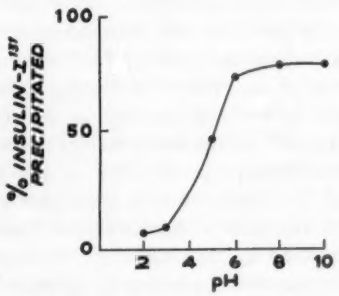

FIG. 4. EFFECT OF pH ON BINDING OF INSULIN-I^{131} TO ANTIBODY.

Figure 3 shows that the addition of 15 per cent urea to the incubation mixtures effectively eliminated almost all of the nonspecific binding in typical serum extracts. Additional salt was required to achieve optimal preferential precipitation. The amount of insulin-I^{131} precipitated by antibody in serum extract was no longer notably different from that in the standard which contained no unlabeled insulin, indicating that the level of circulating human insulin was too low to be detected.

The effect of pH on insulin-antibody complex formation (*Figure 4*). Binding was not detectable below pH 3.0 and reached a maximum at about pH 6. Since insulin and many of the proteins in the serum extracts were insoluble at slightly acid and neutral pH's, pH 8.5 was used for all incubations.

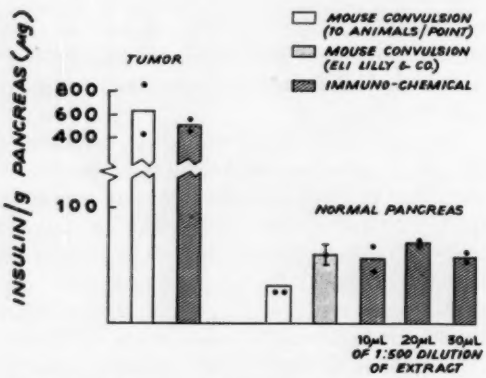

FIG. 5. COMPARISON OF BIOLOGICAL AND IMMUNOLOGICAL ASSAY OF HUMAN INSULIN. Each point represents the mean of duplicate values.

Measurement of human insulin from pancreatic tissue (*Figure 5*). Measurements of human insulin levels in the pancreatic tumor and normal pancreas by biological and immunochemical methods were in good agreement. The extremely good duplication of the assays on normal pancreas by the mouse convulsion method in the Eli Lilly laboratory and by the immunochemical approach was probably coincidental (58.7 μg per g tissue, SD = 4.2 per cent; and 61.5 μg per g tissue, SD = 7.6 per cent, respectively). Results obtained by assaying three different dilutions of the human extract and correcting for dilution differed only within the limits of experimental error. Assay by both methods showed ten times more insulin in the tumor tissue than in the sample of normal pancreas.

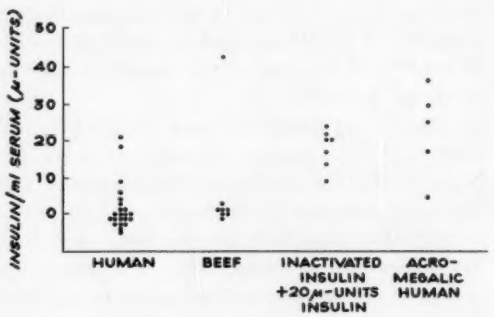

FIG. 6. CIRCULATING LEVELS OF INSULIN IN HUMAN AND BOVINE SERUM. Each point represents the mean of duplicate values.

Circulating level of insulin in human and bovine serum (*Figure 6*). Determination of the circulating insulin in extracts of sera from 22 fasting subjects was attempted. Except on two occasions insulin could not be detected. As seen from the recovery studies on extracts with added insulin, a level of 20 μU per ml of serum is within the minimum sensitivity of this method. Serum insulin levels were notably elevated in four of five subjects with active acromegaly.

The circulating insulin levels in extracts of bovine serum were also determined. This insulin is species identical with the insulin used originally to form the antibodies and to make the standards. In seven of the eight samples tested, as in the human sera, insulin levels were undetectable.

Effects of oral glucose on circulating insulin levels (*Figure 7*). Fifty g of glucose was ad-

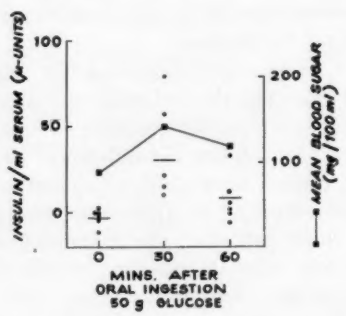

FIG. 7. EFFECT OF ORAL GLUCOSE ON CIRCULATING INSULIN. Each point represents the mean of duplicate values.

ministered orally to six normal fasting subjects. After 30 minutes the serum insulin levels were increased in five of the six subjects (mean, 31 μU). By 60 minutes the insulin levels had commenced to fall and in most instances were no longer detectable. The blood sugar level, elevated at 30 minutes, had also begun to decrease.

Disappearance of crystalline bovine insulin (*Figure 8*). To demonstrate the efficiency of the immunoassay in measuring changing levels of serum insulin, periodic determinations were made after intravenous administration of 10 U of crystalline hormone to a normal subject. Insulin

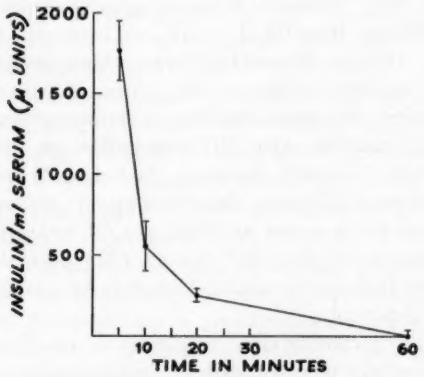

FIG. 8. INSULIN SERUM LEVELS AFTER INJECTION OF CRYSTALLINE BEEF INSULIN (10 UNITS INTRAVENOUSLY) IN NORMAL SUBJECT.

levels were extremely high 5 minutes after the injection; within 20 minutes, however, 90 per cent of the insulin had disappeared. After 60 minutes circulating insulin was no longer detectable.

An insulin-resistant diabetic was given 500 U of insulin (U-500, Eli Lilly & Company) intramuscularly. Twenty-four hours later the serum was collected and extracted to remove the endogenous antibody. Assay of the extract showed a high concentration of insulin (3,100 μU per ml), despite a concomitantly high blood sugar level (301 mg per 100 ml) and severe glycosuria and ketonuria.

DISCUSSION

An immunological method for the measurement of circulating insulin offers the distinct advantage of being chemically specific for the insulin molecule. Growth hormone, epinephrine, steroids, glucagon or tolbutamide do not interfere with the binding of insulin to antibody (12). In the method described in this report the inherent specificity of such techniques is further augmented by the removal of gross impurities from the serum by acid-alcohol extraction. This preliminary step not only removes endogenous insulin antibodies, thereby allowing application of the procedure to serum from individuals previously receiving insulin (11, 12), but also eliminates about 85 per cent of the extraneous serum proteins. Baird and Bornstein (20) have shown that a similar extraction procedure effectively removes many of the globulin inhibitors that plague investigators attempting biological assays. Indeed, preliminary extraction of the serum is of such obvious advantage in increasing specificity that it conceivably could be universally adopted in all insulin assay methods.

The efficiency of extraction with acid alcohol may result in part from the dissociation of insulin antibody complexes noted at acid pH (21). Singer and Campbell (22) have observed that albumin fails to form a precipitating antibody at low pH values. Sri Ram and Maurer (23) suggested that the spontaneous precipitation, rather than affinity of the antigen to the antibody, was the pH-sensitive factor. The insulin-antibody complex, however, is soluble and is quantitated not by spontaneous precipitate formation but by chemical means. The lack of insulin-binding at low pH in this soluble system, as shown by both sodium sulfite and hydrodynamic flow methods (12), indicates the existence of a pH sensitivity at the level of antigen-antibody combination.

The use of salt precipitation to separate free from bound insulin, which is similar to the method described by Farr for albumin (24), requires only a single count of a sharply defined supernatant. In contrast, the hydrodynamic flow method (11, 14) requires additional apparatus for multiple simultaneous determination and, since it distributes the insulin-I^{131} over 10 cm of paper, calls for a higher initial level of radioactivity. Furthermore, at least ten samples must be counted to determine the bound and free peaks, and it is often difficult to differentiate the peaks, particularly in areas of very low or very high binding. Other methods for the preferential isolation of bound insulin have

been reported. Mitchell, Whitehead and O'Rourke (25) used an Amberlite resin for separating the insulin-antibody complex, but noted serious interference by extraneous serum proteins. Skom and Talmage (26) separated the insulin complex from free insulin by precipitating the latter with anti-gamma-globulin sera, a method requiring a second antibody system. Arquilla and Stavitsky (27) utilized a hemolytic technique for the quantitative measurement of insulin-antibody formation, but reported some lack of specificity due to unknown factors affecting red cell lysis.

Nonspecific binding of insulin to serum protein was effectively inhibited by the addition of urea, implying that this phenomenon is caused by hydrogen bonding. On the other hand the comparative ineffectiveness of urea in dissociating the insulin-antibody complex was not surprising, since the affinity of this antigen to its antibody may be primarily due to interatomic van der Waals' forces (12).

The most serious drawback to an insulin assay based on an immunological reaction is the necessity for assuming that the human insulin from extracts and the beef insulin used in the standards have the same capacity for binding to antisera that is produced against beef insulin in guinea pigs. Thus our inability to detect circulating insulin in human sera could be ascribed to its failure to cross react with these antibodies. Although Burrows and associates (15) reported that human insulin did not bind, Moloney and Aprile (21), Arquilla and Stavitsky (28), Berson and Yalow (29) and Wright (30) have independently demonstrated the cross reactivity of human insulin with antibodies produced from beef insulin. The close agreement between our measurements by the immunological and the mouse convulsion assay of human insulin extracted from pancreas indicates that effective cross reactivity does occur. (Exact evaluation of the quantitative relationship between human and beef insulin, however, must await the availability of a crystalline human insulin of sufficient purity to allow unequivocal determination of its insulin content by biological assay.) In addition, a very low level of circulating insulin is not necessarily the result of poor cross reaction, since insulin was not detected in beef blood in which no species difference exists between standards and circulating hormone.

The circulating form of insulin may be different from that extracted from the pancreas. This hypothesis has been suggested by the observation that insulin and the "insulin-like" activity in serum are found in different fractions after chromatographic separation on a Dowex-50 column (31). In the hope of minimizing this problem, we have adopted an acid alcohol procedure for extracting the serum similar to that used for commercial extraction of insulin from pancreas.

Circulating insulin was not detectable by immunochemical assay, although this method is sufficiently sensitive to measure as little as 20 μU of insulin per ml of serum. This level is lower than those estimated from biological techniques, which vary from 65 μU to 13.5 mU per ml (32–35). In most of these techniques, values obtained with *unextracted* serum were compared with results from standards dissolved in various arbitrary media including saline, different buffers or serum albumin. Recently, however, Anderson, Wherry and Bates (36), using extracts of plasma, reported normal levels as low as 25 μU per ml, and Field, Weinberg, Johnson and Spoont (37) have suggested that the circulating level may be less than 10 μU per ml.

The stimulating effect of glucose on insulin secretion was demonstrated by immunological assay. An increase in circulating insulin (mean, 31 μU) was found 30 minutes after oral administration of glucose. The decrease in circulating insulin after 60 minutes was paralleled by a fall in the blood sugar level.

An increase in the serum insulin concentration of patients with acromegaly has been reported by Wright (38) and also by Randle (34), who suggested that the apparent rise may be a reflection of the interference of large amounts of growth hormone in the assay. In contrast, Bornstein and Lawrence (10) not only reported no detectable insulin but described a hyperglycemic factor in blood from acromegalic patients which they believed might be due to glucagon. Since the immunochemical assay is not affected by either growth hormone or glucagon, our results indicate that the level of circulating insulin is truly elevated in acromegalic patients.

After injecting insulin-I^{131} and measuring the trichloroacetic acid-precipitable activity in serum, Elgee, Williams and Lee (39) found that 90 per

cent of the starting level of insulin was cleared from the blood within 15 to 20 minutes. This technique could be subject to error, since part of the circulating radioactivity might have represented partially degraded insulin that was still insoluble in trichloroacetic acid. Others (11, 17), using hydrodynamic flow-electrophoresis to differentiate degraded from unmodified insulin-I^{131}, also noted this rapid clearance. A parallelism between the rate of disappearance of insulin-I^{131} and immunochemically measurable insulin in the rabbit has also been reported (13). These observations, as well as the validity of using insulin-I^{131} as a metabolic tracer, have been confirmed in man by the finding that 90 per cent of the immunologically active insulin was cleared from the circulation in the first 15 to 20 minutes.

The demonstration that resistant diabetics may have high concentrations of insulin in their blood 24 hours after administration of the hormone despite concurrent hyperglycemia, glycosuria and ketonuria agrees with results obtained by biological assays of serum extracts (20, 40). It is apparent that in investigations measuring antibody titers or insulin-binding capacity in resistant serum, the results can be grossly affected by the partial saturation of the serum antibodies by variable unmeasured amounts of endogenous insulin. To minimize this effect, serum should be collected from the donor subjects at the greatest interval possible after insulin administration. A concomitant assay of total insulin would also be useful in this type of study.

Allowing the assumption that insulin-I^{131} and endogenous circulating insulin are extracted from serum in a similar manner, the immunological method for insulin assay described in this report is not affected by antibodies, antagonists or "insulin-like" substances. The method, at present, is applicable to quantities of total insulin above 20 μU per ml of serum. It is expected that increased sensitivity can be achieved by the synthesis of insulin-I^{131} of higher specific activity.

That a portion of the insulin measured by immunological techniques may include biologically inactive precursors or degradation products retaining antigenic activity cannot at present be excluded. Furthermore, as seen in the insulin-resistant diabetic, the total circulating insulin is not always biologically available to the subject. The measurement of "total insulin" in conjunction with the determination of "net active insulin" by biological assays, however, should lead to a greater understanding of the interplay between circulating insulin and its inhibitors, antagonists and "insulin-like" factors in normal and disease states.

SUMMARY

The new method for the immunochemical assay of circulating human insulin described in this report is based on the per cent change in binding of insulin-I^{131} to insulin antibodies in the presence of varying quantities of unlabeled insulin. The degree of binding is measured quantitatively after preferential precipitation of bound insulin with sodium sulfite.

Preliminary extraction of serum with acid alcohol resulted in an 80 per cent recovery of insulin-I^{131} regardless of the presence of endogenous antibodies. The method is therefore applicable to serum from subjects who have previously had insulin therapy.

Nonspecific binding caused by extraneous proteins in the extracts was effectively removed with 15 per cent urea. Affinity of insulin to its antibodies was pH-sensitive, being negligible at acid pH and reaching a maximum at neutral or alkaline pH.

Measurements of insulin extracted from human pancreas by the described chemical method and by mouse convulsion assay were in close agreement, indicating that effective cross reaction occurs between human insulin and antibodies made against beef insulin in the guinea pig.

Circulating levels of human insulin were lower than the minimum sensitivity of the assay (20 μU per ml of serum). Elevated insulin levels were detected in normal subjects after oral administration of glucose and in patients with active acromegaly.

Studies with the immunochemical assay demonstrated a rapid disappearance from serum of injected crystalline bovine insulin, confirming the results of others who employed the clearance of an insulin-I^{131} tracer. Insulin-resistant diabetics still retain large amounts of circulating insulin 24 hours after intramuscular injection of the hormone, despite severe hyperglycemia, glycosuria and ketonuria.

ACKNOWLEDGMENTS

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ADDENDUM

In a recent report published during the final preparation of this manuscript, Yalow, R. S., and Berson, S. A. [Nature (Lond.) 1959, **184**, 1648] have described an immunochemical assay for insulin in unextracted human plasma. These authors reported a fasting circulating level for insulin of 64 to 98 μU per ml, which could be enhanced notably after glucose administration.

REFERENCES

1. Grodsky, G. M. An assay of insulin by fibril formation from small samples of pancreas. Biochem. J. 1958, **68**, 142.
2. Grodsky, G., and Tarver, H. Paper chromatography of insulin. Nature (Lond.) 1956, **177**, 223.
3. Bornstein, J. A technique for the assay of small quantities of insulin using alloxan diabetic, hypophysectomized, adrenalectomized rats. Aust. J. exp. Biol. med. Sci. 1950, **28**, 87.
4. Groen, J., v.d. Geld, H., Bolinger, R. E., and Willebrands, A. F. The anti-insulin effect of epinephrine. Its significance for the determination of serum insulin by the rat diaphragm method. Diabetes 1958, **7**, 272.
5. Winegrad, A. I., Shaw, W. N., Lukens, F. D. W., Stadie, W. C., and Renold, A. E. Effects of growth hormone *in vitro* on the metabolism of glucose in rat adipose tissue. J. biol. Chem. 1959, **234**, 1922.
6. Leonards, J. R. Insulin-like activity of blood. What it is. Fed. Proc. 1959, **18**, 272.
7. Field, J. B. Studies on the circulating insulin inhibitor found in some diabetic patients exhibiting chronic insulin resistance. J. clin. Invest. 1959, **38**, 551.
8. Field, J. B., and Stetten, D., Jr. Humoral insulin antagonism associated with diabetic acidosis. Amer. J. med. 1956, **21**, 339.
9. Vallance-Owen, J., Hurlock, B., and Please, N. W. Plasma-insulin activity in diabetes mellitus measured by the rat diaphragm technique. Lancet 1955, **2**, 583.
10. Bornstein, J., and Lawrence, R. D. Plasma insulin in human diabetes mellitus. Brit. med. J. 1951, **2**, 1541.
11. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K. Insulin-I^{131} metabolism in human subjects: Demonstration of insulin binding globulin in the circulation of insulin treated subjects. J. clin. Invest. 1956, **35**, 170.
12. Grodsky, G. M., Peng, C. T., and Forsham, P. H. Effect of modification of insulin on specific binding in insulin-resistant sera. Arch. Biochem. 1959, **81**, 264.
13. Berson, S. A., and Yalow, R. S. Isotopic tracers in the study of diabetes. Advanc. biol. med. Phys. 1958, **6**, 349.
14. Grodsky, G. M., and Peng, C. T. Extractable insulin measured by immuno-chemical assay: Effect of tolbutamide. Proc. Soc. exp. Biol. (N. Y.) 1959, **101**, 100.
15. Burrows, B. A., Peters, T., and Lowell, F. C. Physical binding of insulin by gamma globulins of insulin-resistant subjects. J. clin. Invest. 1957, **36**, 393.
16. Freund, J., Lipton, M. M., and Thompson, G. E. Aspermatogenesis in the guinea pig induced by testicular tissue and adjuvants. J. exp. Med. 1953, **97**, 711.
17. Scott, G. W., Prout, T. E., Weaver, J. A., and Asper, S. P. A comparison of the behavior of insulin and insulin labeled with I^{131} in serum. Diabetes 1958, **7**, 38.
18. Jephcott, C. M. Extraction of insulin and stability of various preparations. Trans. roy. Soc. Can., Sect. V, 1931, **25**, 183.
19. Hemmingsen, A. M. The accuracy of insulin assay on white mice. Quart. J. Pharm. 1933, **6**, 187.
20. Baird, C. W., and Bornstein, J. Plasma-insulin and insulin resistance. Lancet 1957, **1**, 1111.
21. Moloney, P. J., and Aprile, M. A. On the antigenicity of insulin: Flocculation of insulin-anti-insulin. Canad. J. Biochem. 1959, **37**, 793.
22. Singer, S. J., and Campbell, D. H. Physical chemical studies of soluble antigen-antibody complexes. IV. The effect of pH on the reaction between bovine serum albumin and its rabbit antibodies. J. Amer. chem. Soc. 1955, **77**, 3504.
23. Sri Ram, J., and Maurer, P. H. Modified bovine serum albumin. VII. Studies on the role of free carboxyl groups of the protein in the interaction with its antibodies. Arch. Biochem. 1959, **83**, 223.
24. Farr, R. S. A quantitative immunochemical measure of the primary interaction between I BSA and antibody. J. infect. Dis. 1958, **103**, 239.
25. Mitchell, M. L., Whitehead, W. O., and O'Rourke, M. E. Differential resin binding of insulin in serum. Endocrinology 1959, **65**, 322.
26. Skom, J. H., and Talmage, D. W. Nonprecipitating insulin antibodies. J. clin. Invest. 1958, **37**, 783.
27. Arquilla, E. R., and Stavitsky, A. B. The production and identification of antibodies to insulin and their use in assaying insulin. J. clin. Invest. 1956, **35**, 458.
28. Arquilla, E. R., and Stavitsky, A. B. Evidence for the insulin-directed specificity of rabbit anti-insulin serum. J. clin. Invest. 1956, **35**, 467.

29. Berson, S. A., and Yalow, R. S. Recent studies on insulin-binding antibodies. Ann. N. Y. Acad. Sci. 1959, **82**, 338.
30. Wright, P. H. The effect of insulin antibodies on glucose uptake by the isolated rat diaphragm. Biochem. J. 1959, **71**, 633.
31. Antoniades, H. N., Beigelman, P. M., Pennell, R. B., Thorn, G. W., and Oncley, J. L. Insulin-like activity of human plasma constituents. III. Elution of insulin-like activity from cationic exchange resins. Metabolism 1958, **7**, 266.
32. Martin, D. B., Renold, A. E., and Dagenais, Y. M. An assay for insulin-like activity using rat adipose tissue. Lancet 1958, **2**, 76.
33. Beigelman, P. M., and Antoniades, H. N. Insulin-like activity of human plasma constituents. IV. Insulin levels of normal human serum and plasma. Metabolism 1958, **7**, 269.
34. Randle, P. J. Plasma-insulin activity in acromegaly assayed by rat-diaphragm method; preliminary communication. Lancet 1954, **1**, 441.
35. Willebrands, A. F., and Groen, J. Determination of serum insulin by the rat diaphragm method; further observations in diabetic and nondiabetic subjects and in hyperinsulinism. Diabetes 1956, **5**, 378.
36. Anderson, E., Wherry, F., and Bates, R. W. Factors influencing secretion of insulin. Fed. Proc. 1959, **18**, 4.
37. Field, J. B., Weinberg, A., Johnson, P., and Spoont, S. An unusual case of diabetes mellitus with increased pancreatic production of insulin (abstract). J. clin. Invest. 1959, **38**, 1003.
38. Wright, P. H. Plasma-insulin estimation by the rat-diaphragm method. Lancet 1957, **2**, 621.
39. Elgee, N. J., Williams, R. H., and Lee, N. D. Distribution and degradation studies with insulin-I^{131}. J. clin. Invest. 1954, **33**, 1252.
40. Moloney, P. J., and Coval, M. Antigenicity of insulin: Diabetes induced by specific antibodies. Biochem. J. 1955, **59**, 179.

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STUDIES OF THE VENTILATION–PERFUSION RELATIONSHIPS IN THE LUNGS OF SUBJECTS WITH CHRONIC PULMONARY EMPHYSEMA, FOLLOWING A SINGLE INTRAVENOUS INJECTION OF RADIOACTIVE KRYPTON (Kr^{85}). I. PRESENTATION AND VALIDATION OF A THEORETICAL MODEL *

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The study of the elimination of an inert gas from the lungs of human individuals has proven a useful tool in the determination of ventilation patterns (1, 2). In particular it could be demonstrated that the lungs of emphysematous patients can be conceived as being composed of at least two different compartments, i.e., a relatively small space with good ventilation and a relatively large space with poor ventilation (3).

In an attempt to establish the ventilation-perfusion relationship in the different lung compartments of patients suffering from pulmonary emphysema, Briscoe (4, 5) correlated the ventilation and volume patterns with the oxygen saturation of mixed arterial blood: a very large and poorly ventilated air compartment in the emphysematous lung was shown to be relatively underperfused with blood (6).

In order to check on these findings it was decided to measure the distribution of ventilation and perfusion (especially the latter) in emphysema with an independent technique. Dissolved radioactive krypton (Kr^{85}) was injected intravenously and its rate of disappearance was measured in both expired air and arterial blood. A preliminary report of this work has been published (7). The purpose of this paper is to describe the behavior of intravenously injected krypton in the emphysematous subject and to show how this behavior may be used to determine the distribution of perfusion in the lung. With this objective we present *1*) a theoretical model of the lungs and tissues of the emphysematous man under these conditions; *2*) a mathematical solution of the equations of the three-compartment analog thus depicted; *3*) a comparison of the predicted theoretical curves with the experimental curves which are actually obtained; *4*) three methods for interpreting experimental data in terms of the dimensions, ventilation and blood perfusion of several lung compartments: *a*) an exact method, which might be applicable under optimal conditions to experiments with sampling at three sites, and *b*) two approximate methods, applicable to data limited as to time and number of sampling sites as they usually are in practice.

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† Fellow of the Swiss Academy of Medicine, Fellow in Medicine of Columbia University. Supported by the American-Swiss Foundation for Scientific Exchange.

I. THEORETICAL MODEL: THREE COMPARTMENT ANALOG OF THE BODY IN EMPHYSEMA

The sequence of events which take place after the rapid injection of a dissolved tracer gas of low solubility into a systemic vein of a human subject is best understood if the behavior of a model, essentially analogous to the experimental situation, is studied. The experiment begins with the rapid injection of radioactive Kr^{85} dissolved in saline (8). The usual site of injection is a large vein as near as possible to the heart; the optimal site is the right heart itself which was used in the studies reported here. As the slug of dissolved Kr^{85} passes through the pulmonary capillaries almost all of it diffuses into the alveolar gas spaces because of the relative insolubility of krypton (8). In emphysema these spaces vary greatly in their degree of ventilation. A small part of the lung is well ventilated. That fraction of the injected Kr^{85} which is carried in

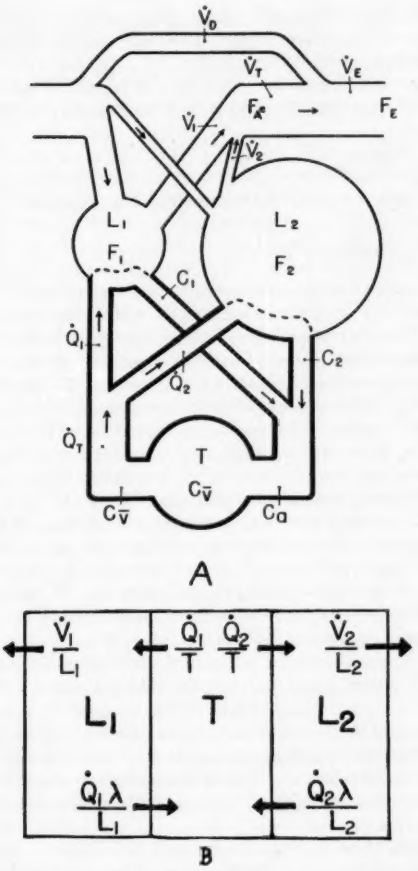

FIG. 1. MODEL SIMULATING THE WASHOUT OF INTRAVENOUSLY INJECTED Kr^{85} IN PATIENTS WITH PULMONARY EMPHYSEMA. For explanation of symbols see text. 1A shows the three chambers under consideration and the flows between them; 1B the fractional rate constants, indicated by arrows, are used in the solution of the differential equations 2, 3 and 4.

the blood to, and deposited in the well ventilated air spaces, is rapidly eliminated in 2 to 5 minutes. A much larger part of the lung is homogeneously very poorly ventilated. The krypton deposited in these air spaces at the time of injection is still being eliminated in appreciable amounts in the gas expired from them 20 minutes later. The end capillary blood leaving both groups of alveoli is considered at all times to contain krypton at the same partial pressure as the alveolar gas. The quantities of krypton in solution in blood are relatively small, compared to the quantities in the gas phase [$\lambda = 0.051$ (9)]. The end capillary blood from the differently ventilated lung spaces mixes in the arterial blood stream which then passes to the tissues. In the first few minutes after injection, arterial blood contains more Kr^{85} than the tissues, and the tissue Kr^{85} concentration rises. As krypton is ventilated out of the lung spaces, arterial Kr^{85} concentration falls. After reaching a peak a few minutes after injection, tissue Kr^{85} concentration falls. Venous Kr^{85} concentration is considered at all times to be equal to that in the tissues. Krypton from the tissues is carried in venous blood to the lungs, redeposited into alveolar gas, and eliminated in ventilation, until eventually no Kr^{85} remains in the body.

The simplest model, therefore, for the quantitative considerations of this situation consists of three reservoirs or mixing chambers, exchanging krypton by means of the blood stream, and with two of these reservoirs eliminating Kr^{85} through ventilatory channels into an infinite space. This model is schematically depicted in Figures 1A and 1B. The three mixing chambers are the well ventilated small lung compartment (L_1), the poorly ventilated large lung compartment (L_2) and the tissue space (T). Three-compartment systems, both closed (10, 11) and open (12–14), have previously been described; the algebraic treatment is therefore abbreviated here. It is noteworthy that in the present model direct access is limited to one compartment (T). The output from the two lung compartments (L_1 and L_2) cannot be measured individually; it is possible, however, to measure their combined output which is reflected in arterial blood and expired air. Arterial blood is the mixture of the blood equilibrated in the two lung compartments, weighted by their respective flows. Mixed expired alveolar gas is the mixture of the gas in the two lung compartments, weighted by their respective ventilations. Expired gas is mixed expired alveolar gas with its krypton diluted by inspired gas from the dead space. In the model calculations the dead space is assumed to be zero, in which case mixed expired alveolar and expired gas are the same. Another specific feature of this model is the combination of a liquid and a gaseous carrier phase. This required knowledge of *a*) the solubility characteristics of the tracer

gas, and *b*) the difference in counting efficiency of liquid and gaseous specimens containing tracer (9).

The description of the emphysematous patient's body in terms of only three compartments did not originate *de novo* but from studies of their behavior. Although there are probably differences among the well ventilated alveoli (L_1) and among the various tissues included in the tissue space (T), it appears from nitrogen washout studies that in severe emphysema there is often a surprising lack of variation among the alveoli in the poorly ventilated group L_2 (6). This group of alveoli constitutes the major reservoir for krypton, and has a dominant influence on the behavior of injected krypton; so it is justifiable, and a very useful simplification, to ignore the variation in L_1 and T and to consider the body as if it consisted of only three compartments.

II. MATHEMATICAL DESCRIPTION OF THE BEHAVIOR OF THE MODEL

Having described the model in physiological terms which characterize the emphysematous patient, our next aim is to construct theoretical curves for comparison with the data actually obtained in patients. Furthermore, these theoretical curves will be analyzed by various approximate methods easily applicable to patients in order to determine the error in these methods. The construction of the curves necessitates some rather lengthy calculations, and before proceeding to the comparison between predicted and observed behavior it is necessary to indicate in outline the assumptions (Section IIA), the algebraic principles (Section IIC), and the numerical values of the coefficients in our examples (Section IID).

A. Assumptions

The theoretical model is applied to experimental situations with the following assumptions:

1. Blood and gas leaving a mixing chamber are in complete equilibrium with this chamber. Deviations from this are believed to be insignificant as regards inert gas exchange in the lung (15, 16) with which this study is mainly concerned. The quantities exchanged in the tissues are of secondary importance and if this assumption does not apply here it has no important effect on our conclusions.

2. Ventilation and perfusion are continuous rather than periodic. There is no rebreathing of Kr^{85} by one lung compartment from the respiratory dead space of gas expired from a differently ventilated lung compartment. This assumption has no important effect on the conclusions when there are large differences between the ventilation of the different lung compartments, as is the case in emphysema. Expired gas is mixed expired alveolar gas diluted by a constant flow of inspired gas, the dead space ventilation. Since this dead space ventilation has no effect on the system except to reduced expired concentrations to a constant fraction of mixed expired alveolar concentrations, it has been considered to be zero in our theoretical cases; as explained later it must be allowed for in practice.

3. The solubility of Kr^{85} (λ) is the same for blood and body tissues.

4. The amount of Kr^{85} in the blood is negligible.

B. Explanation of symbols

Some of the symbols have already been referred to. It is convenient at this point to give a complete list of the symbols used subsequently, with units of each indicated.

Gas volumes (liters): L_1 in well ventilated alveoli; L_2 in poorly ventilated alveoli, $L_T = L_1 + L_2$. *Tissue volume* (liters): T. *Gas flows* (liters per minute): $\dot{V}_1$ in well ventilated alveoli; $\dot{V}_2$ in poorly ventilated alveoli; $\dot{V}_T = \dot{V}_1 + \dot{V}_2$; $\dot{V}_E$ expired ventilation; $\dot{V}_D$ dead space ventilation. *Blood flows* (liters per minute): $\dot{Q}_1$ in well ventilated alveoli; $\dot{Q}_2$ in poorly ventilated alveoli; $\dot{Q}_T = \dot{Q}_1 + \dot{Q}_2$.

Quantities of Kr^{85} (counts per minute): X in L_1, Y in L_2, I injected. *Concentrations of krypton in gas phase* (counts per minute per liter): F_1 in well ventilated alveoli; F_2 in poorly ventilated alveoli; F_E in expired air. F_A° in mixed expired alveolar gas. *Concentration of krypton in blood phases* (counts per minute per liter): $C_1 = F_1\lambda$ in well ventilated alveoli, $C_2 = F_2\lambda$ in poorly ventilated alveoli; C_a in arterial blood; $C_{\bar{v}}$ in mixed venous blood and tissue. $C_{\bar{v}}'$ in mixed venous blood during passage in injectate. *Solubility coefficient at body temperature:* λ. *Time* (minutes): t = time in general, u = time spent by blood in alveolar capillary; t_p = time of maximal concentration in mixed venous blood. The symbol (0) signifies boundary conditions at zero time.

With these assumptions and symbols in mind, the behavior of the analog model can be expressed in the following way.

C. Algebraic formulation

1. Rate of change of krypton concentration: differential equations. Considering compartment L_1 as an example, the following relationship holds for each instant:

$$\frac{dX}{dt} = \frac{d(L_1F_1)}{dt} = (\dot{Q}_1C_{\bar{v}} + \dot{V}_1 0) - (\dot{Q}_1F_1\lambda + V_1F_1). \quad [1]$$

Rearranging and using rate constants in Figure 1B, Equation 1 and the similar equations for L_2 and T become:

$$\frac{dF_1}{dt} = \frac{\dot{Q}_1}{L_1}C_{\bar{v}} - \frac{\dot{Q}_1\lambda}{L_1}F_1 - \frac{\dot{V}_1}{L_1}F_1 \quad [2]$$

$$\frac{dF_2}{dt} = \frac{\dot{Q}_2}{L_2}C_{\bar{v}} - \frac{\dot{Q}_2\lambda}{L_2}F_2 - \frac{\dot{V}_2}{L_1}F_2 \quad [3]$$

$$\frac{dC_{\bar{v}}}{dt} = \frac{\dot{Q}_1\lambda}{T}F_1 + \frac{\dot{Q}_2\lambda}{T}F_2 - \frac{\dot{Q}_1}{T}C_{\bar{v}} - \frac{\dot{Q}_2}{T}C_{\bar{v}}. \quad [4]$$

This system of three simultaneous linear differential equations, each being homogeneous, of the first order, and

with constant coefficients, can be solved by classical methods to yield a solution in the following form (17):

$$F_1 = {}^1C_1e^{m_1t} + {}^1C_2e^{m_2t} + {}^1C_3e^{m_3t} \quad [5]$$

$$F_2 = {}^2C_1e^{m_1t} + {}^2C_2e^{m_2t} + {}^2C_3e^{m_3t} \quad [6]$$

$$C_{\bar{v}} = {}^3C_1e^{m_1t} + {}^3C_2e^{m_2t} + {}^3C_3e^{m_3t}. \quad [7]$$

The exponents m_1, m_2, m_3 are the three roots of a cubic equation and can all be computed (e.g., by Newton's method) from the numerical values of $\dot{V}_1$, $\dot{V}_2$, $\dot{Q}_1$, $\dot{Q}_2$, L_1, L_2, T and λ. To determine the values of the constants (C's) in Equations 5, 6 and 7, it is necessary to know also the initial values $F_1(0)$, $F_2(0)$ and $C_{\bar{v}}(0)$ of the krypton concentrations in the three compartments.

2. Initial krypton concentrations: boundary conditions, $F_1(0)$, $F_2(0)$. These were determined by the following approach. It is assumed that the injectate reaches the lungs as a homogeneous square front slug which takes u minutes to pass through the alveoli. The value of u was taken to be 0.2 minute. Its exact value has no important effect on our conclusions.

During this time the mixed venous concentration of krypton is constant at $C_{\bar{v}}'$, the prime indicating that we are dealing with the period during which boundary conditions are established.

Before the end of the passage of the injectate through the lungs, the tissue concentration of Kr^{85} ($C_{\bar{v}}$) will have begun to rise. However, this rise is very small and it greatly simplifies the situation to regard it as negligible. In that case $C_{\bar{v}}(0) = 0$, after the passage of the injectate through the lungs.

The equations which apply during the passage of the injectate through the lungs are the same as Equations 2, 3 and 4, except that the variable $C_{\bar{v}}$ (from tissues) is replaced in all three equations by the constant $C_{\bar{v}}'$ (from injectate), so that Equation 4 becomes $dC_{\bar{v}}'/dt = 0$.

During the time u, F_1 and F_2 rise from zero to $F_1(u)$ and $F_2(u)$ at the end of the passage of the injectate through the lungs. By integration of Equations 2 and 3 under these conditions one has:

$$F_j(u) = \frac{\dot{Q}_jC_{\bar{v}}'}{\dot{V}_j + \dot{Q}_j\lambda}\left[1 - e^{-u(\dot{V}_j+\dot{Q}_j\lambda)/L_j}\right] \quad [8]$$

where the subscript j is either 1 or 2. These concentrations $F_1(u)$ and $F_2(u)$ occurring at the end of the passage of the slug are the peak values of F_1 and F_2, and are used as boundary conditions $F_1(0)$ and $F_2(0)$ in numerical examples discussed subsequently. The initial concentration in the tissues $C_{\bar{v}}(0)$ is zero.

It is relevant to our later discussion of the slow krypton method to consider what fraction (θ) of the krypton brought to the alveoli in the blood is lost in the expired gas and the blood which leaves during the passage of the injectate. The krypton remaining in the alveoli at the end of the passage of the injectate is $1 - \theta$.

$$\theta_j = \frac{\dot{Q}_jC_{\bar{v}}'u - F_j(u)L_j}{\dot{Q}_jC_{\bar{v}}'u} = 1 - \frac{1 - e^{-u(\dot{V}_j+\dot{Q}_j\lambda)/L_j}}{u(\dot{V}_j + \dot{Q}_j\lambda)/L_j} \quad [9]$$

in which $\dot{Q}_jC_{\bar{v}}'u$ is the quantity of Kr^{85} brought to these alveoli by the blood, and $F_j(u) = F_j(0)$. Figure 2 illustrates the values of θ for alveoli of different quantities of ventilation and perfusion per unit lung volume. In the case of the "slow" alveoli, θ is about 0.02. This concludes the discussion of the boundary conditions.

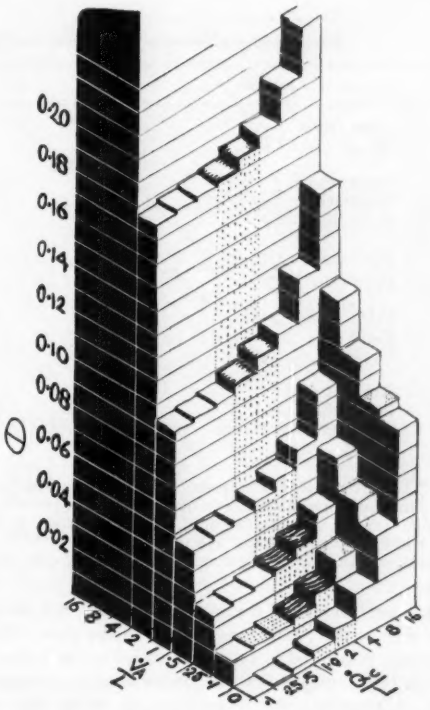

FIG. 2. THE RELATION BETWEEN ALVEOLAR VENTILATION, ALVEOLAR PERFUSION, LUNG VOLUME AND THE FRACTION OF THE INJECTED KRYPTON REMAINING IN THE ALVEOLI AFTER PASSAGE THROUGH THE LUNG OF THE INJECTATE. θ is the fraction of the injected Kr^{85} which is lost from the alveoli in expired gas and end capillary blood during the passage of the injectate. This passage occupies time u, which is 0.2 minute in this case. The diagram shows how much θ changes with variation in blood perfusion per unit lung volume ($\dot{Q}_c/L$) and alveolar ventilation per unit lung volume ($\dot{V}_A/L$). The four blocks whose tops are shaded apply to conditions in L_2 and indicate that in this case θ = 1 to 3 per cent.

When I, $\dot{Q}_1$, $\dot{Q}_2$, L_1, L_2, $\dot{V}_1$, $\dot{V}_2$, T and u are known, then $F_1(0)$ and $F_2(0)$ can be determined.

3. Krypton concentrations resulting from admixture: mixing equations. Mixed expired alveolar gas is formed by admixture from L_1 and L_2 and its krypton concentration is:

$$F_{A^c} = \frac{\dot{V}_1F_1 + \dot{V}_2F_2}{\dot{V}_1 + \dot{V}_2}. \quad [10]$$

Arterial blood is also formed by admixture,

$$Ca = \frac{\dot{Q}_1\lambda F_1 + \dot{Q}_2\lambda F_2}{\dot{Q}_1 + \dot{Q}_2}. \quad [11]$$

TABLE I

*Parameters and boundary conditions used for the calculation of the three numerical examples**

Numerical example		I	II	IIIa	IIIb
Lung space	L_1	1	1	1	1
(*liters*)	L_2	4	4	4	4
Tissue space	T	60	10	60	60
Ventilation	$\dot{V}_1$	4.5	4.5	4.5	4.5
(*L/min*)	$\dot{V}_2$	0.5	0.5	0.5	0.5
Perfusion	$\dot{Q}_1$	2	2	1.4	1.4
(*L/min*)	$\dot{Q}_2$	3	3	3.6	3.6
$F_1(0)$ (*cpm/L*)		131,000	131,000	[260.98 = $F_1(10)$	15.61 = $F_1(60)$]†
$F_2(0)$		74.000	74,000	[16,542 = $F_2(10)$	268.7 = $F_2(60)$]
$C_{\bar{v}}(0)$		0	0	[598.8 = $C_{\bar{v}}(10)$	35.42 = $C_{\bar{v}}(60)$]
Amount of Kr^{85} injected at zero minus u (= 0.2) min (*cpm*)			500,000		

* The parameters are assumed round figures which are typical of patients with severe chronic pulmonary emphysema. The boundary conditions have been calculated from the amount of Kr^{85} injected (I = 500,000 counts per minute), its passage time, u, through the pulmonary capillaries (u = 0.2 min), and the parameters for examples I and II.

† In examples IIIa and IIIb the boundary conditions are concentrations, respectively, 10 or 60 minutes after injection. These are indicated by the symbols F_1 (10), F_2 (10), $C_{\bar{v}}$ (10), and F_1 (60), F_2 (60), $C_{\bar{v}}$ (60).

Thus the behavior of the analog model, i.e., the changes with time of the Kr^{85} concentrations in the three mixing chambers, in arterial blood and in mixed expired alveolar gas can be completely described in terms of its parameters (size of chambers, exchange rates of carrier between chambers) and the boundary conditions. Mixed expired gas is mixed expired alveolar gas diluted by the admixture of dead space ventilation. Therefore, the mixed expired krypton concentration is:

$$F_E = F_A \cdot \frac{\dot{V}_1 + \dot{V}_2}{\dot{V}_1 + \dot{V}_2 + \dot{V}_D} \qquad [12]$$

where $\dot{V}_1 + \dot{V}_2 + \dot{V}_D = \dot{V}_E$. $\dot{V}_D$ is constant in any study and must be determined by other methods (18).

D. Numerical examples calculated from the analog model

Three numerical examples have been calculated in detail.

1. It was of interest to know how the model behaves if the naturally occurring situation is imitated as closely as possible. The values chosen for the parameters $L_{1,2}$, $\dot{V}_{1,2}$ and $\dot{Q}_{1,2}$ are typical of those found by other methods in patients with severe chronic pulmonary emphysema (6). These parameters are listed in Table I, (numerical examples I and II).

2. The volume of tissues, however, which equilibrates with Kr^{85} in such patients is not known, even as an approximation. An attempt was, therefore, made to assess the magnitude of the possible effect of the tissue volume on the washout of Kr^{85} by considering two extreme situations:

TABLE II

*Numerical values of the exponents, m, and the constants of integration, C, of Equations 6, 7 and 8 calculated from the figures listed in Table I**

Numerical example	I	II	IIIa	IIIb
m_1	−4.6007	−4.6049	−4.5703	
m_2	−0.1814	−0.5260	−0.1943	
m_3	−0.0636	−0.1315	−0.0586	
$^1C_1$	130,996.7	130,960.0	77.215	4.5823
$^1C_2$	−706.90	−1,232.488	−83.013	0.1307
$^1C_3$	709.868	1,272.158	266.777	10.8969
$^2C_1$	8.157	54.168	0.0040	0.00024
$^2C_2$	61,777.3	5,180.114	9,593.67	−15.1144
$^2C_3$	12,214.4	68,765.82	6,948.32	283.814
$^3C_1$	−48.273	−320.85	−0.019	−0.00117
$^3C_2$	−1,561.73	−2,510.58	−259.455	0.4087
$^3C_3$	1,610.11	2,842.32	859.66	35.114

* The units of the constants are counts per minute per liter, and of the exponents, $minutes^{-1}$.

in one numerical example (no. I) a value of 60 L was arbitrarily chosen for the tissue volume T, whereas in the second numerical example (no. II) T was assumed to be 10 L (see Table I).

3. It has been shown that the blood flow supplying the poorly ventilated lung space in emphysematous subjects increases by about 20 per cent when 100 per cent O_2 is breathed instead of room air (7). In order to confirm this finding, a number of studies was made in which the patient was switched from room air to pure oxygen as the inspired gas in the middle of a Kr^{85} washout. It was expected that a 20 per cent change in the distribution of perfusion to the lung would be reflected in a detectable alteration of the washout pattern. In the numerical example III, we study this in the analog model by assuming that an instantaneous 20 per cent increase of $\dot{Q}_2/\dot{Q}_T$ occurs 10 minutes (example IIIa) and 1 hour (example IIIb), respectively, after the injection of Kr^{85}. The parameters which apply to this situation are again listed in Table I (numerical examples IIIa and IIIb). Using the figures listed in Table I the numerical values for the constants of integration and the exponents m_1, m_2 and m_3 of Equations 5, 6 and 7 have been computed (Table II). Finally, the values of F_1, F_2, $C_{\bar{v}}$, $F_A{}^e$, and C_a have been computed for times varying from 0 to 120 minutes after injection. The corresponding curves are shown in Figures 3, 4 and 5.

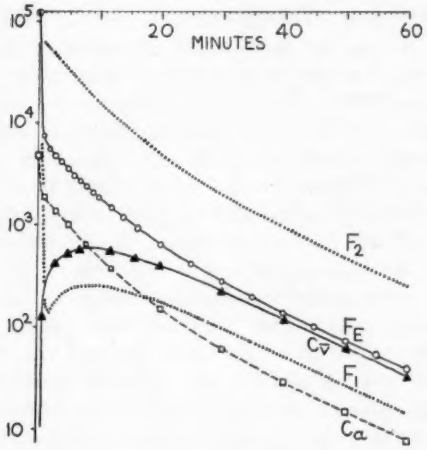

FIG. 3. THE WASHOUT OF KRYPTON FROM THE MODEL IN EXAMPLE I WITH TISSUE VOLUME (T) 60 L. Symbols are the same in Figures 3 through 7. Circles, expired air-concentration, (F_E); squares, arterial blood concentration, (C_a); triangles, mixed venous blood, ($C_{\bar{v}}$). In Figures 3 and 4 the krypton concentration in the well ventilated (F_1) and poorly ventilated (F_2) alveoli are indicated by dotted lines. Ordinates in Figures 3, 4 and 5 are counts per minute per liter on a logarithmic scale. In these same figures the dead space ventilation ($\dot{V}_D$) is assumed to be zero, so that $F_E = F_A{}^e$. When dead space ventilation is not zero all values of F_E are reduced: $F_E = F_A{}^e(\dot{V}_E - \dot{V}_D)/\dot{V}_E$, and would lie in a line below but parallel to the F_E lines in Figures 3, 4 and 5. The curves for F_1, F_2, C_a, $C_{\bar{v}}$ are not affected by the magnitude of the dead space ventilation.

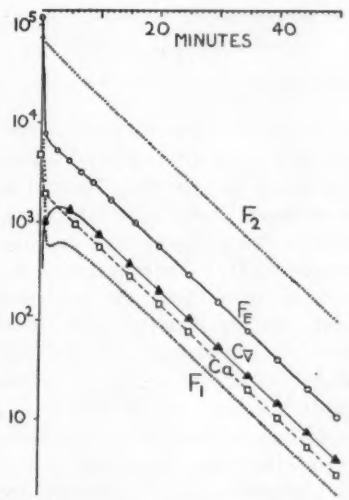

FIG. 4. WASHOUT OF Kr^{85} FROM THE MODEL IN EXAMPLE II, WITH TISSUE VOLUME (T) 10 L. The venous peak is earlier than in Figure 1 and compares with Figures 5 and 6.

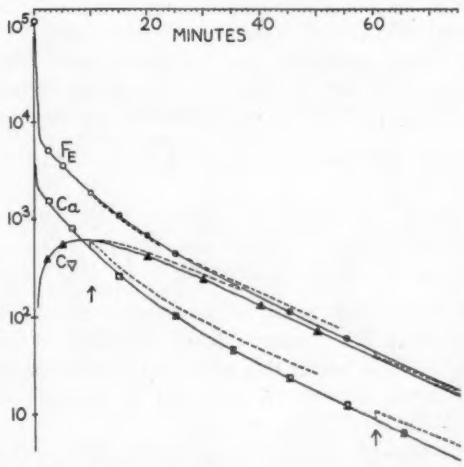

FIG. 5. WASHOUT OF Kr^{85} IN THE MODEL III IN WHICH THE DISTRIBUTION OF PERFUSION CHANGES ABRUPTLY AT 10 MINUTES (IIIA) OR AT 60 MINUTES (B). The modified curves of F_E, C_a and $C_{\bar{v}}$ resulting from this are indicated by dashed lines. The main effect of this change in the distribution of perfusion is on C_a.

III. COMPARISON OF THEORETICAL AND EXPERIMENTAL CURVES

A. Theoretical curves

As can be seen in Figures 3 and 4, both mixed expired air and mixed arterial blood behave similarly up to about 15 minutes. Thereafter, however, the washout in example I becomes much more delayed; this is due to the large size of the tissue compartment. The concentration of Kr^{85} is highest at all times, except the first few seconds in the poorly ventilated alveoli (L_2). In the first few seconds the concentration in the well ventilated alveoli (F_1) may exceed this if its blood flow ($\dot{Q}_1$) is large in relation to its volume (L_1). F_1 falls steeply due to its large relative ventilation ($\dot{V}_1/L_1$) and then rises at about two minutes when Kr^{85} returns in larger amounts from the tissues ($C_{\bar{v}}$). However, these events in F_1 occur at insignificant concentrations, and concern insignificant amounts of Kr^{85}, when compared to the much larger concentrations and quantities of Kr^{85} in other chambers at the same time.

F_E always exceeds C_a; in Figures 3 and 4 these lines run approximately parallel courses. Both these parameters are formed by admixture, so F_E must always lie between F_1 and F_2, and C_a between C_1 and C_2.

The venous and tissue concentration ($C_{\bar{v}}$) is initially zero and rises rapidly to a peak. It then falls gradually back toward zero. The quantity of Kr^{85} entering the tissues before the peak is equal to the total quantity leaving the tissues after the peak, i.e., $\dot{Q}_T \int_0^{t_p} (C_a - C_{\bar{v}}) \cdot dt = \dot{Q}_T \int_{t_p}^{\infty} (C_{\bar{v}} - C_a) \cdot dt$, where t_p is the time of the peak, or more generally: $\int_0^{\infty} (C_a - C_{\bar{v}}) \cdot dt = 0$. The peak of venous concentration occurs at the crossover point where $C_a = C_{\bar{v}}$. The time at which this peak occurs depends on the tissue volume; it is earlier in example II with the small tissue volume.

In example I, with a large tissue volume, this dominates the rate of the latter stages of washout. $C_{\bar{v}}$ exceeds C_a by much more than it does in example II. There is an inflection in the lines in example I after 20 minutes which is not seen in example II. F_1 exceeds C_a in example I but not in example II, due to this cause. The partial pressure in the tissues exceeds that in L_2 by a considerable amount in the late stages of example I, but not in example II. (Equal partial pressures occur in Figures 3, 4 and 5 when $F_2 = 20\ C_{\bar{v}}$.)

B. Experimental curves

It is instructive to consider the situation which is found to occur in fact when Kr^{85} is injected into patients with emphysema.

Most of the experimental data which have been obtained so far were based on curves of Kr^{85} decay in expired air and arterial blood after injection of 300μc (7). A few studies are available, however, with the more complete protocol which included mixed venous blood. Examples are given in Figures 6 and 7 which should be compared with Figures 3 and 4. In Case I, 1,000 μc of Kr^{85} was injected through a cardiac catheter into the pulmonary artery.[1] Another cardiac catheter was used to sample venous blood. This catheter was in the superior vena cava. In Case II, 600 μc of Kr^{85} was injected into the right ventricle through one catheter, while the other catheter sampled mixed venous blood in the right atrium. Samples were taken at the times indicated in Figures 6 and 7 from the brachial artery and from collections of mixed expired air, as well as from the cardiac catheter. The two subjects (Figures 6 and 7) resemble the model example I in the approximately parallel course taken by C_a and F_E. The wider separation of these in the subjects is due to the higher counting efficiency of our equipment for gases, which is not allowed for in the model curves. They also resemble the model in the times at which the various exponents dominate the F_E curve. The first rapid exponent has no apparent effect after about 5 minutes. The third exponent does not dominate until after 20 minutes have elapsed.

Subject I (Figure 6) shows resemblance both to example I (Figure 4) and example II (Figure 5). As in example I, he has a prolonged slow washout, presumably from tissues, which indi-

[1] The tracheal mucosa, which is the tissue most irradiated, receives a dose of 0.7 rad after the intravenous injection of 1,000 μc of Kr^{85}. This small dose is calculated on the basis of a biological half-life of 10 minutes.

cates that some tissues which took up Kr^{85} are perfused by a relatively small blood flow. Like example II, he shows an early venous concentration peak, but this peak does not coincide with the crossover point of the arterial and venous concentration curves. When mixed venous blood is sampled, it is mathematically inevitable that the peak coincides with the crossover point. The inconsistency here arises from the position of the sampling catheter in the superior vena cava. Case II is given here, although the smaller injected dose results in low and therefore

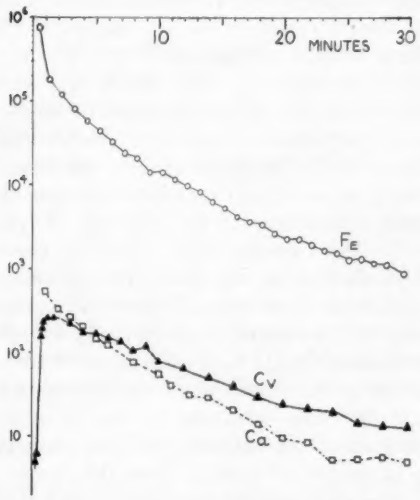

FIG. 6. WASHOUT OF Kr^{85} IN CASE I, WITH CHRONIC PULMONARY EMPHYSEMA. The venous sampling catheter was in the superior vena cava. The venous peak is at 2 minutes. The C_a and $C_{\bar{v}}$ lines cross later (see text). In Figures 6 and 7 the ordinates are counts per minute on a logarithmic scale. In blood, 1 mμc per ml gives 70 cpm; in gas, 1 mμc per ml gives 6,200 cpm.

erratic counts later in the study, to show that when mixed venous blood is sampled inside the heart, the peak and crossover points do in fact coincide. Furthermore, the peak occurs early, as in example II, rather than late, as in example I.

These considerations suggest that when dealing with the earlier points of the curve (0 to 15 minutes), the model with the small effective tissue volume (example II) is more applicable, whereas in the late stages (60 minutes), the model with the large tissue volume (example I)

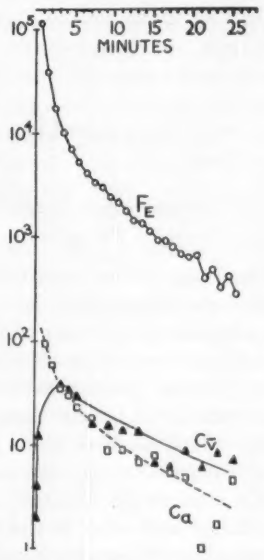

FIG. 7. WASHOUT OF Kr^{85} IN CASE II WITH CHRONIC PULMONARY EMPHYSEMA. The venous peak is at 3 minutes and coincides with the crossover point of the C_a and $C_{\bar{v}}$ lines. The venous sampling catheter was in the right auricle. The later C_a and $C_{\bar{v}}$ points are scattered because of the low activity after a relatively small injectate (see text).

is closer to the observed facts. At least two tissue compartments with different blood flows per unit volume would be needed to fit an early peak which occurred with a slow rate of late fall in $C_{\bar{v}}$ as is suggested by the lines drawn among the scattered points in Figure 7.

TABLE III

*Effects of changes in the distribution of perfusion upon the ratio of Kr^{85} concentration between arterial blood (C_a) and the expired air (F_E)**

Time after injection	$\dot{Q}_2/\dot{Q}_T$ unchanged	$\dot{Q}_2/\dot{Q}_T$ increased by 20%	
	C_a/F_E	C_a/F_E	Increase
min		A†	%
10	0.265	0.318	20
12	0.258	0.320	24
50	0.205	0.273	33
		B†	
60	0.204	0.241	18
62	0.204	0.268	31
100	0.204	0.273	34

* Dead space ventilation is zero, so $F_E = F_A$.
† A, at 10 minutes; B, at 60 minutes.

Be that as it may, the data in Figures 6 and 7 show reasonable agreement with the three-compartment model and the imperfections and irregularities inevitable in data of this type do not warrant any more involved system of interpretation at present.

C. Influence of instantaneous changes in the distribution of perfusion upon the curves

The principal changes occurring in the washout pattern when the distribution of perfusion is altered instantaneously (increase of $\dot{Q}_2/\dot{Q}_T$ by 20 per cent) are summarized in Table III. The most striking change takes place in the mixed arterial blood: its Kr^{85} concentration increases instantaneously by 20 per cent if the distribution of perfusion changes at 10 minutes and by 18 per cent if $\dot{Q}_2/\dot{Q}_T$ rises at 60 minutes. An equally important change will occur in the ratio of the Kr^{85} concentrations of simultaneously collected arterial blood and gas specimens. However, the changes in the F_E and $C_{\bar{v}}$ curves are slight, as can be seen in Figure 5. This illustrates the important point that changes in the distribution of perfusion can be detected only by the arterial blood composition, or by some ratio related to this, such as C_a/F_E.

IV. METHODS FOR THE ANALYSIS OF EXPERIMENTAL CURVES TO DERIVE RELATIVE VENTILATION AND/OR PERFUSION OF COMPARTMENTS

So far we have been considering the behavior of a theoretical three-compartment model. We have chosen arbitrary values for its parameters and tried to predict the changes with time of Kr^{85} in the three compartments. The ultimate aim of a washout study is, of course, reciprocal: we must analyze the experimental data and we want to determine from such an analysis as many parameters as possible (i.e., in our case, volumes, ventilation and perfusion).

Such an analysis will vary depending on whether the experimental protocol provides access to all three possible sampling sites—expired air, arterial and mixed venous blood (which entails sampling from the right heart)—or whether access is limited to expired air and arterial blood. First, a method is indicated which might be used under optimal conditions. Second, two approximation methods are described which have been widely used for the evaluation of actual experimental data. Finally, an attempt is made to assess the possible error introduced by the use of approximation formulae.

A. Exact analysis for curves obtained under optimal conditions: sampling from three sites—$C_{\bar{v}}$, C_a and F_E

The task in this case is to analyze the three experimental curves for F_E, C_a and $C_{\bar{v}}$. Since the $C_{\bar{v}}$ curve represents the output from one single compartment, its conventional analysis (10) should readily yield the numerical values of its constants and exponents, i.e., ${}^3C_1$, ${}^3C_2$, ${}^3C_3$ and m_1, m_2 and m_3. This, however, can only yield useful values when the data show less scatter and continue for longer periods than is the case in Figures 6 and 7, since the first stage in the analysis is the exact determination of the slowest component of the washout in venous blood. The analysis of the F_E and C_a curves is complicated by the fact that they each represent a mixture of the contents of more than one compartment. Nevertheless, a method for deriving the values of $\dot{Q}_1$, $\dot{Q}_2$. $\dot{V}_1$, $\dot{V}_2$, L_1, L_2 and T from the analysis of the curves has been developed.[2] It would be very laborious to use in practice. Furthermore, the values derived are most sensitive to the rate of washout from the tissues, and are dependent on the assumption that there is only one tissue compartment.

B. Sampling from two sites for a limited time—C_a and F_E: approximation methods

If catheterization of the right heart cannot be performed, no representative specimen of mixed venous blood can be obtained and sampling is confined to mixed arterial blood and mixed expired air. Furthermore, in practice, studies are limited in time. When 300 μc, which was the usual dose, is injected C_a and F_E are too low for accurate counting with our equipment about 20 minutes after injection.

The slow krypton method for determining $\dot{Q}_2/\dot{Q}_T$. This depends on the notion that the fate of the injected Kr^{85} is determined in the branches of

[2] A description of this method of analysis, which was developed with the help of Mr. Boss, will be sent by the authors on request.

the pulmonary artery a few seconds after injection, and just prior to its passage through the lung. Those Kr^{85} molecules which are directed to the well ventilated alveoli pass into the alveolar gas and are rapidly eliminated in the expired air in the next two or three minutes. Those molecules which are directed to the poorly ventilated alveoli remain much longer in alveolar gas. They are slowly eliminated in the expired air from the poorly ventilated alveoli and to a lesser extent from the well ventilated alveoli which they reach after transfer to and temporary storage in the tissues. An assessment of this quantity which is slowly eliminated is made as follows.

It can be seen from Figure 7 (experimental data) that, when data for F_E are available for only 20 minutes they resemble, within the limits of experimental error, a washout with only two exponents, m_4 and m_5, and two constants, C_4 and C_5:

$$F_E = C_4 e^{m_4 t} + C_5 e^{m_5 t}. \qquad [13]$$

The values for m_5 and C_5 can be determined by the slope and the intercept at zero time of a line drawn through the data between 5 and 20 minutes. These constants do not necessarily have any simple relationship to those numbered 1, 2 and 3. There is no need for such algebraic formulation since they are used merely to derive an integral: $\dot{V}_E \cdot C_5 \int_0^\infty e^{m_5 t} \cdot dt$. This quantity of krypton which is slowly eliminated is considered as an approximation to consist of those molecules which were present in L_2 at zero time, i.e.,

$$F_2(0)\, L_2 = \dot{V}_E C_5 \int_0^\infty e^{m_5 t} \cdot dt.$$

Furthermore, it is shown in Figure 2 that θ is about 2 per cent in the slow alveoli, i.e., 98 per cent of the Kr^{85} which reached these alveoli in the blood during the passage of the injectate is present in the alveolar gas at the end of this passage. Thus $L_2 \cdot F_2(0) = I \cdot \dot{Q}_2/\dot{Q}_T$ where I is the total quantity of Kr injected. Therefore, $\dot{Q}_2/\dot{Q}_T$ can be estimated by the relationship:

$$\frac{\dot{Q}_2}{\dot{Q}_T} = \frac{\dot{V}_E C_5 \int_0^\infty e^{m_5 t} \cdot dt}{I} \qquad [14]$$

where I is the total quantity injected or the total quantity eliminated in expired gas in infinite time. This approximation was tested by analyzing the data over the period 5 to 20 minutes in examples I and II in each of which $\dot{Q}_2/\dot{Q}_T = 0.60$. In example I, $\dot{Q}_2/\dot{Q}_T$ was estimated at 0.524, an error of −12.6 per cent. In example II the estimate was 0.609, an error of +1.5 per cent.

The partition coefficient method for determining $\dot{Q}_2/\dot{Q}_T$. Like the slow krypton method, this method of assessing $\dot{Q}_2/\dot{Q}_T$ depends on data collected in studies limited in time to 15 or 20 minutes. In this case data for both C_a and F_E are used. It is evident from study of examples I and II that in the period from between 5 and 20 minutes after injection, the Kr^{85} which was initially present in the fast alveoli, $L_1F_1(0)$, has been eliminated. The only Kr^{85} in L_1 is that brought by the blood from the tissues. During this period F_1 is small compared to F_2, and may be regarded as negligible. If F_1 is zero then the only source of Kr^{85} in C_a and F_E in this period, from 5 to 20 minutes, is L_2. In that case the following approximate relationship can be used to determine $\dot{Q}_2/\dot{Q}_T$:

$$\frac{F_E}{C_a} = \frac{\dot{V}_2}{\dot{V}_E \lambda} \div \frac{\dot{Q}_2}{\dot{Q}_T} \qquad [15]$$

in which C_a and F_E are simultaneous measurements at a time between 5 and 20 minutes after injection, and $\dot{V}_2/\dot{V}_E$ is known by use of another technique (N_2 washout curve). Applying this method to examples I and II in which $\dot{Q}_2/\dot{Q}_T = 0.60$ we have estimates of 0.543 and 0.524 for $\dot{Q}_2/\dot{Q}_T$. They are in error by −9.5 and −12.6 per cent, respectively.

Thus, these two arbitrary assessments of $\dot{Q}_2/\dot{Q}_T$, the slow krypton method and the partition coefficient method, have errors in these examples comparable to the errors of other physiological assessments in man.

SUMMARY

1. The behavior of intravenously injected Kr^{85} in the emphysematous patient is considered in terms of a model composed of three compartments: well ventilated alveoli, poorly ventilated alveoli, and tissues.

2. The differential equations applicable in

this model have been solved. Using typical emphysematous values for the volume, ventilation and blood flows of the two lung compartments, the behavior of Kr^{85} in the various gases and blood streams is predicted when the effective tissue volume is either 10 or 60 L. Further calculated examples show the predicted effect of changes, during a study, in the distribution of blood flow to the lungs.

3. Data are presented on the findings in two patients with emphysema in whom Kr^{85} concentrations were followed in expired air, arterial blood and mixed venous blood, after intracardiac injection of Kr^{85}. The behavior of the patients resembled that predicted for the model; the differences are discussed.

4. A method has been developed in which, by analysis of simultaneous expired, arterial and mixed venous concentration curves, the distribution of perfusion to the lung could be assessed. It is laborious in use and of very doubtful validity. Two approximate methods for determining the distribution of perfusion in the lung are presented. One depends only on expired gas analysis and the other on expired gas and arterial blood analysis. When tested on the predicted curves these appear to be in error by about 10 per cent.

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REFERENCES

1. Robertson, J. S., Siri, W. E., and Jones, H. B. Lung ventilation patterns determined by analysis of nitrogen elimination rates; use of the mass spectrometer as a continuous gas analyzer. J. clin. Invest. 1950, **29**, 577.
2. Jones, H. B. Respiratory system: Nitrogen elimination *in* Medical Physics, O. Glasser Ed. Chicago, Year Book Publishers, 1950, vol. II, p. 855.
3. Fowler, W. S., Cornish, E. R., and Kety, S. S. Lung function studies. VIII. Analysis of alveolar ventilation by pulmonary N_2 clearance curves. J. clin. Invest. 1952, **31**, 40.
4. Briscoe, W. A. A method for dealing with data concerning uneven ventilation of the lung and its effects on blood gas transfer. J. appl. Physiol. 1959, **14**, 291.
5. Briscoe, W. A. Comparison between alveolo-arterial gradient predicted from mixing studies and the observed gradient. J. appl. Physiol. 1959, **14**, 299.
6. Briscoe, W. A., Cree, E. M., Filler, J., Houssay, H. E. J., and Cournand, A. Interrelationships between lung volume, alveolar ventilation and perfusion in chronic pulmonary emphysema. J. appl. Physiol. In press.
7. Briscoe, W. A., Hardewig, A., Emmanuel, G., Gurtner, H. P., Rochester, D., and Cournand, A. The distribution of blood perfusion to the lung in emphysema. Fed. Proc. 1959, **18**, 16.
8. Chidsey, C. A., III, Fritts, H. W., Jr., Hardewig, A., Richards, D. W., and Cournand, A. The fate of radioactive krypton (Kr^{85}) introduced intravenously in man. J. appl. Physiol. 1959, **14**, 63.
9. Hardewig, A., Rochester, D. F., and Briscoe, W. A. Measurement of solubility-coefficients of krypton in water, plasma and human blood, using radioactive Kr^{85}. J. appl. Physiol. In press.
10. Robertson, J. S. Theory and use of tracers in determining transfer rates in biological systems. Physiol. Rev. 1957, **37**, 133.
11. Robertson, J. S., Tosteson, D. C., and Gamble, J. L., Jr. The determination of exchange rates in three-compartment steady-state closed systems through the use of tracers. J. Lab. clin. Med. 1957, **49**, 497.
12. Steele, R. Retention of metabolic radioactive carbonate. Biochem. J. 1955, **60**, 447.
13. Skinner, S. M., Clark, R. E., Baker, N., and Shipley, R. A. Complete solution of the three-compartment model in steady state after single injection of radioactive tracer. Amer. J. Physiol. 1959, **196**, 238.
14. Lewallen, C. G., Berman, M., and Rall, J. E. Studies of iodoalbumin metabolism. I. A mathematical approach to the kinetics. J. clin. Invest. 1959, **38**, 66.
15. Forster, R. E. Exchange of gases between alveolar air and pulmonary capillary blood: Pulmonary diffusing capacity. Physiol. Rev. 1957, **37**, 391.
16. Kety, S. S. Theory and application of exchange of inert gas at lungs and tissues. Pharmacol. Rev. 1951, **3**, 1.
17. Ayers, F. Theory and Problems of Differential Equations. New York, Schaum Publishing Co., 1952.
18. Fowler, W. S. Lung function studies. II. The respiratory dead space. Amer. J. Physiol. 1948, **154**, 405.

ALDOSTERONE SECRETION AND PRIMARY AND MALIGNANT HYPERTENSION *

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After the synthesis of desoxycorticosterone (DOC), it became apparent that this corticosteroid, the physiological effects of which are concerned primarily with sodium and potassium metabolism, can produce a state of hypertension both in animals and in man (1, 2). The induced hypertension is dependent upon the administration of adequate amounts of dietary sodium, whereas the hypertensive state, produced by administration of glucocorticoids such as cortisone, differs in that it is independent of the dietary sodium content (3).

More recently, following the chemical and biological characterization of the mineralocorticoid hormone, aldosterone (4), a disease state associated with primary hypersecretion of this hormone has been described in man (5). Aldosterone produces effects on sodium and potassium metabolism similar to those of desoxycorticosterone, and the clinical syndrome of primary aldosteronism resembles the disease state produced by chronic administration of DOC to dogs (6). Arterial hypertension has been a consistent finding in patients with primary hyperaldosteronism.

A number of other observations have suggested a relationship between the dietary sodium intake and the blood pressure level of patients with primary (benign essential) hypertension. The beneficial effects of sodium deprivation, and of various natriuretic agents in certain patients with hypertension, is well known. In addition, other studies have suggested that abnormalities of intracellular sodium and potassium content may occur in patients with arterial hypertension (7). Genest, Koiw, Nowaczynski and Lebouef (8) have reported that the urinary excretion of aldosterone was significantly increased in 55 per cent of their patients with hypertension of diverse etiologies. The increased levels of urinary aldosterone were not correlated with any abnormality of sodium or potassium metabolism, nor did the increased values appear to be specifically associated with any particular type of hypertensive disease.

It has been recognized that the measurement of urinary excretion of unchanged hormone may not accurately reflect significant changes in adrenal secretion of aldosterone. During the past several years a method has been devised which provides an estimate of the amount of aldosterone which is actually produced by the adrenal glands (9). The technique involves the injection of a trace amount of tritiated aldosterone and the determination of the specific activity of a urinary metabolite of aldosterone in the subsequent 24 hour urine. The difference between the specific activity of the injected hormone and that of the urinary metabolite is considered to be a measure of the endogenous production of aldosterone and, as such, permits the estimation of the daily secretion rate. In the present report, this technique has been applied to the study of patients with arterial hypertension. In the course of these studies an effort has been made to correlate abnormalities in aldosterone secretion with abnormalities in sodium and potassium metabolism and, when possible, with changes in the morbid anatomy of the adrenal gland itself. The results have been compared with values obtained from normal subjects and from patients with known primary aldosteronism.

METHODS AND MATERIALS

Patient selection. All of the patients studied were hospitalized. A complete history and physical examina-

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tion, routine laboratory tests, plasma and urinary electrolytes, intravenous pyelography and measurement of catecholamine excretion were obtained in all patients. None of the patients included in the group had evidence of congestive heart failure. Frequently, the dietary intake of sodium was stabilized by means of a constant diet prepared by the metabolic ward kitchen, and serial control observations were then made while the patients were on the metabolic ward. These detailed studies will be reported at a later date. Some of the patients were hospitalized at the Goldwater Memorial Hospital, Columbia Division. All of the latter group had been in the hospital for considerable periods of time, and in several instances, daily blood pressure measurements for several years, as well as frequent determinations of plasma and urinary electrolytes, were available.

Classification of hypertensive patients. After efforts to exclude primary renal or adrenal disease the remaining hypertensive group selected for study was subdivided. The patients with elevated blood pressures but with no evidence of renal insufficiency or of arteritis were classified as "primary hypertension" (often called, "benign essential" hypertension). The patients with high blood pressure and evidence of renal or retinal complications were classified as "advanced hypertension" or "primary hypertension with complications." The criterion for renal complications was the presence of either proteinuria or a blood urea nitrogen greater than 30 mg per 100 ml or both. Retinal complications refer to the presence of retinal hemorrhages (Grade III retinopathy according to the standard classification). Patients with unequivocal papilledema were classified as the accelerated form or "malignant" hypertension. It was recognized that this classification, although useful, was arbitrary, and that diverse etiologies could exist within these categories. For comparative purposes a group of patients with primary aldosteronism was also studied. Diagnosis in these patients was made on the basis of *1*) hypokalemic alkalosis, *2*) hypertension, and *3*) the finding of an adrenal tumor at operation or at postmortem.

The metabolic ward techniques and the methods used for measurement of plasma and urinary electrolytes have been reported previously (10). Blood pressures were measured with a standard cuff-type mercury manometer.

The *d*-aldosterone employed in this study was tritiated by the Wilzbach procedure (11) in the laboratories of the New England Nuclear Corporation. It was then purified to constant specific activity by paper chromatography. The paper used in the final chromatogram was washed in a Soxhlet apparatus with ethanol for 1 week prior to use. The aldosterone was eluted from this chromatogram with ethanol, and the eluate was filtered through a fine sintered glass filter. This solution was diluted with sterile water to make a 10 per cent ethanol solution for intravenous injection.

Urine was collected for 24 hours after the injection of tritiated aldosterone and hydrolyzed with β-glucuronidase at pH 5.0 for 3 days at 37° C. An extract of the hydrolyzed urine was prepared with ethyl acetate. The tetrahydro-metabolite of aldosterone was isolated from the extract by paper chromatography, and acetylated with C^{14}-labeled acetic anhydride of known specific activity. The triacetate of the metabolite then was chromatographed first on paper and next on a partition column. The H^3/C^{14} ratios in the fractions from the partition column were determined by liquid scintillation counting with a Packard Tri-Carb scintillation counter using the discriminator ratio method of Okita, Kabara, Richardson and LeRoy (12). Constancy of the ratios in the area of the tritium peak is indicative of radiochemical purity. The aldosterone secretion rate in micrograms per day can be shown to equal

$$\frac{\text{counts per minute of aldosterone injected}}{\text{specific activity of the metabolite in 24 hour urine (counts per minute per microgram)}}$$

H^3/C^{14} times the specific activity of the C^{14}-acetic anhydride equals the specific activity of the aldosterone metabolite. It is evident that the H^3/C^{14} ratio is a measure of the specific activity of the metabolite. This technique has been previously outlined (9) and will be described in detail in a manuscript which is in preparation.

Plan of study. Because aldosterone secretion varies considerably with the dietary sodium intake (9), an effort was made to stabilize the dietary sodium content for at least 5 days prior to study. With this precaution and in the absence of any demonstrable renal or adrenal insufficiency it was felt that the 24 hour urinary sodium level represented a reasonably good index of sodium metabolism at the time the aldosterone production was measured. In 17 subjects in whom a metabolic balance was not feasible the urinary sodium content was checked for several days before and after the administration of aldosterone. If these values were not grossly reduced (below 20 mEq per day), if the patients exhibited no other signs of fluid retention, and if there was no history of recent sodium deprivation, a state of sodium retention was considered unlikely. However, in most (20 of 27) of these patients a complete metabolic balance was carried out. In no instance did the patients receive diuretic agents for at least 3 weeks prior to study.

RESULTS

The results are summarized in Table I and in Figure 1.

Normal subjects. In Figure 1 the results obtained in eight normal subjects on unselected diets are presented graphically. The aldosterone secretion rate ranged from 150 to 330 μg per day in these eight subjects. The corresponding 24 hour urinary sodium outputs ranged from 82 to 254 mEq per day. With the same techniques employed as in the present study, it has been shown previously that normal subjects studied increased their aldosterone secretory rate to about 1,000

μg per day in response to sodium deprivation, and that the feeding of large amounts of sodium reduced the rate to as low as 50 μg per day (9).

Primary hypertension. In eight subjects with primary hypertension the adrenal secretory rate of aldosterone ranged from 180 to 330 μg per day. These values are within the normal range. In this group, there were no abnormalities in either plasma or urinary electrolytes suggestive of aldosteronism. All but one of these subjects (no. 2) were without medication. Prior to the study, two of the subjects (nos. 2 and 7) had had at least one cerebral thrombosis with recovery. There was no evidence of renal failure nor of any other complications in the subjects included in this group.

Unilateral renal disease. Patients 9 and 10 manifested the clinical picture of advanced hypertension which was presumably the result of unilateral renal disease. In Patient 9 the diagnosis had been established by demonstration of anomalous right renal arteries on aortography and was confirmed by surgical removal of the abnormal right kidney and a subsequent sharp fall in blood pressure which has now been sustained for a year. A filling defect in one kidney was clearly demonstrated by pyelography in Patient 10 three years after the onset of severe hypertension. This patient was considered a poor surgical risk, and nephrectomy was not performed. Aldosterone secretory rates of Patients 9 and 10 were within the

TABLE I

Aldosterone secretory rates and related clinical and morphological findings in hypertensive patients

Patient no. and diagnosis*	Age and sex	Degree and duration of hypertension B.P.†	Study date	Aldosterone secretion rate	Urine†			Plasma†				Urea	Pathology and remarks
					Na	K	Cl	Na	K	CO_2	Cl		
				μg/day	mEq/day			mEq/L				mg/100 ml	
1													
Primary hypertension	55 F	250/140 10 yrs 200/120	2/24/58	180	35	72	45	137	4.3	23	100	15	
2													
Primary hypertension	60 M	200/130 16 yrs 135/85	3/19/58	330	47	40	54	143	4.5	30	98	18	On hydralazine and reserpine; had had multiple "strokes"
3													
Primary hypertension	40 M	250/120 10 yrs 190/120	7/9/58	180	196	81	133	138	4.3	25	102	15	
4													
Primary hypertension	53 M	190/122 9 yrs 180/110	3/12/59	320	138	49		134	3.8	25	103	22	
5													
Primary hypertension	38 F	220/110 6 yrs 190/110	4/11/59	320	59	63		138	4.5	25	100	17	
6													
Primary hypertension	17 F	240/140 13 yrs 220/120	9/2/58	250	34	43	96	138	4.2	25	100	15	
7													
Primary hypertension	50 F	180/120 6 yrs 170/110	4/2/59	190	103	81	114	134	4.7	24	107	12	Had one small "stroke" and diabetes
8													
Primary hypertension	55 F	220/110 20 yrs 210/100	5/15/59	330	101	152		144	4.2	28	99	18	
9													
Unilateral renal disease	12 M	270/240 11 yrs 225/150	9/14/58 (Before nephrectomy)	200	66	45	57	140	4.3	25	105	16	Anomalous right renal arteries; Grade I fundi (had sympathectomy in 1947) 155/110 post-nephrectomy.
10													
Unilateral renal disease	58 M	198/113 3 yrs 195/110	4/16/59	340	188	76	194	130	3.1	27	97	25	On chlorothiazide, reserpine and hydralazine

* Patient 12 was studied through the courtesy of Dr. Robert D. Gittler; Patient 13, courtesy of Dr. Marion Isaacs of the Veterans Administration Hospital, First Avenue at 24th Street; Patient 14, courtesy of Dr. H. Chase of Brooklyn Jewish Hospital. Patients 20 and 26 were studied through the kind cooperation of Dr. Milford Fulop of the Albert Einstein College of Medicine. Patient 27 has been previously reported in detail (14).

† At time of study.

TABLE I—*Continued*

Patient no. and diagnosis*	Age and sex	Degree and duration of hypertension B.P.†	Study date	Aldosterone secretion rate	Urine†			Plasma†				Urea	Pathology and remarks
					Na	K	Cl	Na	K	CO_2	Cl		
				μg/day	*mEq/day*			*mEq/L*				*mg/100 ml*	
11													
Primary aldosteronism	65 F	200/135 35 yrs 180/110	5/11/58	650	54	30		138	2.0	44	89	20	Right adrenal adenoma, left adrenal atrophy; arteriolar nephrosclerosis moderately advanced
12													
Primary aldosteronism	42 F	205/110 15 yrs 180/110	7/15/58	510	58	54	45	141	2.5	37	107	15	Left 2.5 cm adrenal cortical adenoma removed; no polyuria
13													
Primary aldosteronism	43 M	240/120 10 yrs 220/120	7/7/58	1,350	160	71		140	2.3	24	110	15	Adrenal adenoma 1 inch yellow; microscopic not unusual; CO_2 never high; B.P. 160 to 180/110 postop.; K normal; GFR slightly low
14													
Primary aldosteronism	47 F	180/110 10 yrs 180/110	9/5/58	585	37	70		140	2.6	38	90	18	No polyuria; 3.5 cm tumor removed from right adrenal
15													
Primary aldosteronism	17 F	250/150 5 yrs	2/9/59	1,000	43	74		144	2.5	31	107	19	Right adrenal gland removed (normal gland); left gland had tumor 1.5 cm removed later (7/1/59)
		190/130	3/3/59	1,690 (After right adrenalectomy)	64	68		148	3.2	33	101	14	
16													
Primary hypertension with complications	63 M	240/110 12 yrs 155/75	3/19/58	450	102	44	70	143	3.5	28	103	50	Hydralazine fundi grade III; proteinuria; decreased urea clearance; stroke 1949
17													
Primary hypertension with complications	56 F	220/125 12 yrs 145/80	3/19/58	600	48	29	55	141	3.4	35	91	45	Hydralazine and reserpine; fundi grade III for 5 yrs; died in uremia
18													
Primary hypertension with complications	56 M	155/100 7 yrs 155/105	4/16/59	960	87	61	103	138	3.7	28	100	37	One kidney nonfunctioning for 1 yr by pyelogram calcified pineal; had stroke 2 yrs ago and thrombosis of retinal artery
19													
Primary hypertension with complications	58 M	170/100 4 yrs 170/100	4/16/59	510	115	57	133	130	4.4	25	101	31	Hydralazine and hydrochlorothiazide; proteinuria grade III retinopathy
20													
Primary hypertension with complications	43 F	250/140 3 yrs 250/140	6/16/58	1,170	80	51	61	138	3.5	31	96	18	Bilateral grade III retinopathy; normal glands at exploration; left gland removed; postop. correction of slight plasma alkalosis; persisted hypertension 8 mos later of 180/110
		170/110	9/20/58	350 (Postop.)	150	75	163	138	4.5	26	100	18	
21													
Primary hypertension with complications	47 M	240/130 18 yrs 190/100	7/27/59	130	99	44		138	4.0	26.3	104	36	Mild diabetes
22													
Primary hypertension with complications	51 M	210/120 6 yrs 160/100	7/30/59	240	85	49		142	3.9	27.7	100	17	Hemorrhages in fundi noted 1954, albuminuria +
23													
Primary hypertension with complications	33 F	220/120 4 yrs 190/110	7/15/59	575	137	57		140	5.0	26	103	25	Malar rash 1954 and arthralgia, at times +++ albuminuria, L. E. prep. negative

TABLE I—*Continued*

Patient no. and diagnosis*	Age and sex	Degree and duration of hypertension B.P.†	Study date	Aldosterone secretion rate	Urine†			Plasma†				Urea	Pathology and remarks
					Na	K	Cl	Na	K	CO_2	Cl		
				μg/day	*mEq/day*			*mEq/L*				*mg/100 ml*	
24													
Malignant hypertension	57 F	240/149 12 yrs 160/110	9/13/58	520	130	67	122	138	3.6	30	99	53	Mecamylamine and reserpine
25													
Malignant hypertension	56 M	250/150 20 yrs 240/150	4/28/58	600	69	60	40	135	3.5	35	92	27	Not helped by bilateral adrenalectomy in 2 stages; terminal uremia; adrenal glands showed nodular hyperplasia but normal weight, proteinuria ++
			6/9/58	760 (Postop. first stage)	36	62	30	138	3.2	35	92	41	
26													
Malignant hypertension	46 F	250/150 9 yrs 215/130	3/19/58	800	26	56	42	133	3.5	30	92	35	Reserpine
27													
Malignant hypertension	55 M	240/160 2 mos 250/150	4/21/58	1,020	47	66		139	3.7	28	99	58	Bilateral adrenal cortical hyperplasia at postmortem
28													
Malignant hypertension	59 M	170/100 4 yrs 165/100	4/16/59	600	35	45	40	131	3.9	25	105	30	Hydralazine and reserpine; was alkalotic at times; was severely malignant prior to drug therapy (4 yrs ago)
29													
Malignant hypertension	48 M	280/160 3 yrs 230/130	4/21/59	860	53	79	72	135	3.7	26	95	71	Adrenal glands overweight and hyperplastic at postmortem
30													
Malignant hypertension	53 M	220/130 4 yrs 160/110	5/15/59	1,220	101	54	55	132	3.6	30	92	37	Hydralazine and mecamylamine
31													
Malignant hypertension	42 F	250/160 5 yrs 240/150	3/21/59	2,180	43	66		140	4.1	27	95	45	Adrenal glands normal weight; possibly hyperplasia
			3/26/59	1,650	8.5	50		138	4.4	31	99	54	
			4/8/59	1,450	122	55		133	2.9	27	100	40	
32													
Malignant hypertension	44 F	250/150 15 yrs 200/100	10/8/58	2,730	51	113	91	141	3.2	28	101	47	Microscopic adrenal hyperplasia (nodular)
33													
Malignant hypertension	23 F	260/150 5 yrs 240/150	9/28/58	1,600	67	54	75	125	3.8	32	76	81	Had sympathectomy 8/56; adrenals "normal" at operation
34													
Malignant hypertension	7 F	300/150 5 yrs 250/140	8/30/58	740	36	20	33	133	3.1	28	93	15	Normal aortogram
35													
Malignant hypertension	39 F	260/140 4 yrs 230/130	9/18/58	190	141	47	92	135	4.2	22	102	20	Hydralazine, had sympathectomy 1 yr prior to test with considerable benefit
36													
Malignant hypertension	53 M	250/149 14 yrs 240/140	6/30/59	1,500	25	35		140	3.2	32	101	22	Polyuria 2 to 3 L for 12 yrs
37													
Malignant hypertension	46 F	250/160 12 yrs 220/160	7/3/59	10,000	42	76		126	3.9	27	79	78	Plasma bicarbonate as high as 40 mEq/L, K as low as 2.1; glands grossly normal at post but hyperplastic microscopically
38													
Malignant hypertension	27 M	240/140 6 mos 240/140	7/25/59	700	94	101		135	3.5	30	89	25	Many retinal hemorrhages; ++ albuminuria

normal range, 200 and 340 μg per day, respectively.

Primary aldosteronism. Patients 11–15 represent examples of proven primary aldosteronism (Conn's syndrome). In these patients the adrenal secretory rate was significantly increased above normal values, ranging from 510 to 1,690 μg per day. These elevated aldosterone values were found in the face of relatively high values for urinary sodium excretion. All of these patients ex-

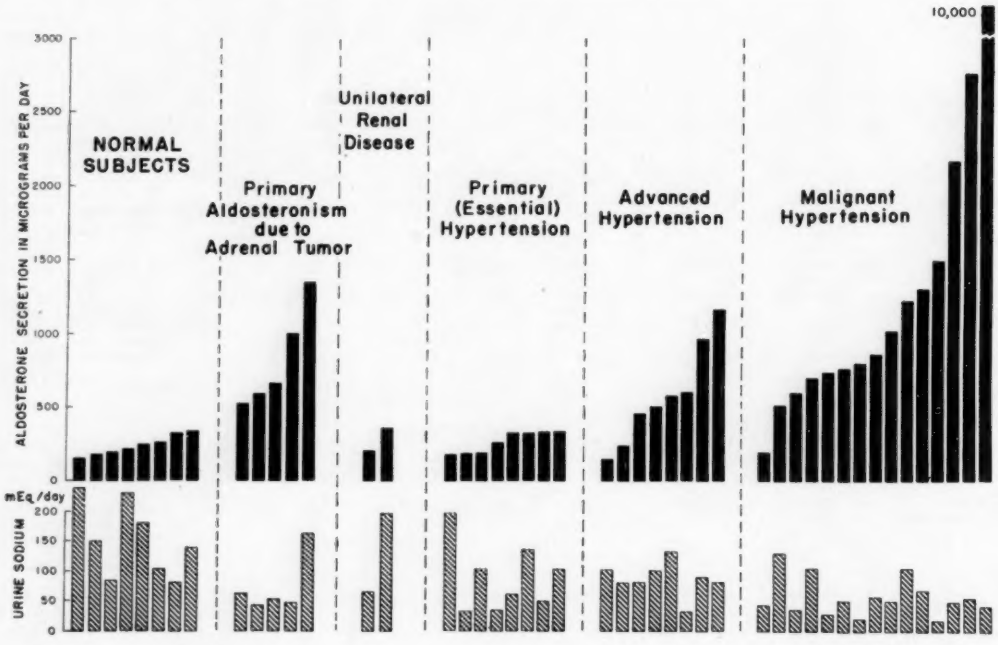

FIG. 1. ADRENAL SECRETION RATES OF ALDOSTERONE (IN MICROGRAMS PER 24 HOURS) FOR 8 NORMAL SUBJECTS, 8 PATIENTS WITH PRIMARY HYPERTENSION, 8 PATIENTS WITH PRIMARY HYPERTENSION WITH COMPLICATIONS ("ADVANCED" HYPERTENSION), AND 15 PATIENTS WITH MALIGNANT HYPERTENSION. For comparative purposes 2 patients with unilateral renal disease and 5 patients with primary aldosteronism are presented. The urinary sodium content of the corresponding 24 hour urine collection is also shown. There is no evident hypersecretion of aldosterone in primary hypertension. In malignant hypertension marked hypersecretion of aldosterone was found and the values were often higher than those encountered in patients with primary aldosteronism. The patients with malignant hypertension resembled those with primary aldosteronism in that the hypersecretion was not associated with grossly reduced sodium excretion.

hibited hypokalemia and all but one had an elevated plasma bicarbonate level. All of these patients had adrenal adenomas. In Patient 11 the tumor was found at autopsy after a negative laparotomy. In Patients 12–15 the symptomatology was relieved by surgical removal of an adrenal adenoma. Patient 15, a girl, aged 17, exhibited severe hypertension with papilledema. This patient was improved after a two-stage total adrenalectomy. An adenoma of the right adrenal gland was found, but all of the adrenal tissue appeared hyperplastic on microscopic study. The significance of the adenoma is thus questionable. Precise classification of this patient is, therefore, difficult, since, as will be seen, she could also have been classified as malignant hypertension with adrenal hyperplasia.

Primary hypertension with complications (Patients 16–23). The patients included in this group had had their hypertensive disease for periods of from 3 to 18 years. Three of these eight patients were on antihypertensive medications as indicated (Table I) at the time of the study. Five of the patients included in this group had significant nitrogen retention (nos. 16–19 and 21) and seven had retinal hemorrhages (nos. 16, 17, 19–23). The aldosterone secretion rates ranged from 130 to 1,170 μg per day in these patients. In five of these eight the values were above 500 μg per day and appeared significantly elevated above normal. As in the patients with primary aldosteronism, the increased aldosterone production was observed in the presence of relatively high urine sodium levels (Figure 1). The plasma bicarbonate levels were 28 mEq per L or higher in four of these patients, and in all but one plasma potassium levels

were less than 4 mEq per L. With one exception (Patient 19) the increased aldosterone secretory rates were found in the patients with relatively high plasma bicarbonate levels. In five of the eight patients in this group the blood urea nitrogen exceeded 30 mg per 100 ml. However, the elevated aldosterone production did not appear to be constantly associated with this nitrogen retention.

Patient 20 was subjected to laparotomy, and, when no tumor was found, a left adrenalectomy was carried out. The gland was grossly normal but microscopically revealed some nodularity and focal hyperplasia. It is of interest that following this procedure there was a sustained (9 months' observation) fall in blood pressure to levels of 170/110 and complete clearing of retinal hemorrhages. After the unilateral adrenalectomy the aldosterone secretion rate was also markedly reduced from 1,170 μg per day to a value within the normal range of 352 μg per day. The sequence of events raised the possibility that hypersecretion of aldosterone might have been a factor in accelerating this patient's hypertensive disease.

Malignant hypertension (Patients 24–38). Fifteen patients with malignant hypertension characterized by papilledema were studied. The known duration of the hypertension in this group ranged from 2 months to 20 years. However, the duration of the papilledema in these cases is difficult to determine. In most instances, it appeared to be a relatively recent development as the disease state progressed from the benign to the so-called accelerated form.

The adrenal secretion rate of aldosterone in these patients with malignant hypertension ranged from 190 to 10,000 μg per day. All of the values were markedly elevated above normal except for that of Patient 35. The range for the remaining 14 patients was from 520 to 10,000 μg per day. Patient 35 differs perhaps from the rest of the group in that at the time of study she was considerably improved following sympathectomy. Furthermore, more recently, papilledema has subsided, and this patient appears to be free of the "malignant" state. Another sympathectomized patient in the group (no. 33) exhibited an elevated secretion rate, but in this patient the operation was not associated with clinical improvement.

In this group of patients with malignant hypertension, significant nitrogen retention was present in 9 of the 15 (blood urea nitrogen over 30 mg per 100 ml). Despite this evidence of renal insufficiency, in 6 of the 15 the plasma bicarbonate values were 30 mEq per L or higher, and in 9 of the 15 the values were 28 mEq per L or higher. These values appear particularly impressive because they were often encountered in the presence of nitrogen retention, which is characteristically associated with retention of fixed anions and an associated reduction in the plasma bicarbonate concentration. Perhaps even more striking was the fact that in 13 of the 15 patients in this group the plasma potassium values were found to be less than 4.0 mEq per L, and in 10 of 15 were less than 3.8 mEq per L. Again, in the face of renal insufficiency, the tendency to hypokalemic alkalosis in these patients is noteworthy and perhaps affords supporting evidence suggesting the presence of hyperaldosteronism.

The markedly increased aldosterone secretion in this group of patients was found, as in the patients with Conn's syndrome, to occur in the presence of relatively high urinary sodium levels and was, therefore, clearly not associated with a state of progressive sodium retention. Although the urinary Na levels were never grossly reduced (range, 25 to 113 mEq per day), they were somewhat lower than those in the other patients (see Figure 1). There was no evidence of significant positive sodium balance in any of these patients and the lowered excretions were the result of lower intake. The increased aldosterone secretion appears to be far in excess of what one would normally find in association with such levels of urinary sodium excretion.

Relation of aldosterone secretion to blood pressure level. No correlation was evident between the degree of elevation of blood pressure and the degree of elevation in the aldosterone secretory rate in this series of patients. Many of the patients with primary hypertension manifested blood pressure elevations which were as high or higher than those observed in the patients with primary hypertension with complications, or in the patients with malignant hypertension.

Relation of aldosterone secretion rate to renal insufficiency. The patients with advanced or with malignant hypertension often exhibited significant nitrogen retention (13 of 23 with blood urea ni-

trogen above 30 mg per 100 ml). Yet, it is difficult to relate the increased aldosterone secretion specifically to this renal failure since a number of the patients (7 of 23) had elevated aldosterone secretion rates with a normal blood urea nitrogen.

It has been shown that patients with sodium-losing renal disease may excrete large amounts of aldosterone. Presumably this is a secondary mechanism in an attempt to conserve sodium. Also, these patients may continue to excrete increased amounts of aldosterone long after sodium repletion, a phenomenon which has been termed "secondary primary aldosteronism" (13). It is conceivable that certain of the patients reported here had antecedent or subtle forms of sodium-losing renal insufficiency, but, insofar as is possible, metabolic balance studies appear to have excluded this possibility at the time of the study in Patients 11–15, 20, 21, 23–28, 31, 34 and 35. These patients all had urinary sodium values of less than 10 mEq per day after dietary deprivation. Patient 13 with primary aldosteronism and Patient 32 with malignant hypertension, manifested slight impairment of renal sodium conservation on low sodium intakes (urine sodium did not fall below 20 mEq per day). Yet, even in these two patients, there was no evidence that the impairment of renal function had preceded the hypersecretion of aldosterone. Therefore, while it is theoretically possible that impaired renal sodium conservation could have preceded and provoked secondary hypersecretion of aldosterone in these hypertensive subjects, the available data do not establish that this was the sequence of events.

It is important to emphasize that, conceivably, as a consequence of severe renal disease the method as employed might yield an apparently high secretory rate in the absence of true hypersecretion of aldosterone. A basic requirement which must be fulfilled, if the isotope dilution technique used in this study is to measure the true secretion rate, is that the reactions involved in the conversion of radioactive hormone to radioactive metabolite and also in excretion of the radioactive metabolite into the urine must have gone to completion within the period during which the urine is collected. In other words, regardless of the other metabolites to which the injected labeled hormone may be converted, all of the isotopically labeled tetrahydrometabolite which is to be formed must have been formed and excreted within the collection period. If additional amounts of radioactive metabolite were to be excreted into subsequent urine collections, then the conditions necessary for determination of the secretion rate by isotope dilution would not exist and the value of the secretory rate derived from such an incomplete collection would be artificially high.

It has already been shown (14) that the fraction of radioactivity originating from injected C^{14}-cortisol that appears in urine and, to a much lesser extent, the rate at which it appears in the urine, are related to the clearance of creatinine. Normal individuals with clearances of about 125 ml per minute excreted about 80 per cent of C^{14} in the first day's urine, whereas patients with kidney disease and creatinine clearances of 42 to 55 ml per minute excreted about 50 per cent in the first day, and patients with creatinine clearances of 5 to 9 ml per minute excreted only about 3 per cent in the first 24 hours. Perhaps more importantly, in all of the subjects, very little additional radioactivity appeared on the second and third days.

The condition set forth above does not require that a certain percentage of injected radioactivity must appear in the urine. If the assumption is made that the injected labeled molecules have the same fate as the endogenously produced hormone, then if only a small fraction of injected hormone is converted to radioactive metabolite, a similar small fraction of the endogenous hormone undergoes the same fate. It is only necessary that the collection period be long enough to permit complete formation and then complete excretion of all the radioactive metabolite which is to be excreted.

Analysis of the data presented in Table II reveals no consistent relationship between the blood urea nitrogen and the aldosterone secretion rate. In Table II seven patients with good renal function (three normal subjects and four subjects with benign hypertension) are compared with seven patients with nitrogen retention. The patients with elevated blood urea values, as a group, excreted less of the injected hormone as the urinary tetrahydrometabolite than did the patients with normal renal function (average of 6.1 compared to 12 per cent). There is overlap in the two

TABLE II

Relationship of blood urea nitrogen concentration to excretion of labeled aldosterone

Patient	Diagnosis	BUN*	Per cent of injected counts released by glucuronidase hydrolysis (24 hour urine)	Aldosterone secretion rate
		mg/100 ml		*μg/24 hrs*
B	Normal	16	14.3	334
F	Normal	18	7.9	170
G	Normal	15	8.7	255
4	Primary hypertension	22	13.5	321
6	Primary hypertension	15	12.0	250
7	Primary hypertension	12	8.5	190
8	Primary hypertension	18	12.1	330
18	Primary hypertension with complications	37	4.7	960
26	Malignant hypertension	35	3.4	800
29	Malignant hypertension	71	5.3	860
30	Malignant hypertension	37	4.2	1,225
31	Malignant hypertension	45	4.3	2,180
32	Malignant hypertension	47	11.5†	2,730
37	Malignant hypertension	78	9.2	10,000

* Blood urea nitrogen.

† In this subject no radioactivity was released by enzyme hydrolysis of the urine collected on the second day after injection.

groups; the former ranging from 7.9 to 14.3 per cent; the latter, from 3.4 to 11.5 per cent. Patient 37 with a blood urea nitrogen of 78 mg per 100 ml had a secretory rate of 10,000 μg per day, whereas Patient 29 with a blood urea nitrogen of 71 mg per 100 ml had a secretory rate of only 860 μg per day.

Of the patients in this group an effort was made only once to determine whether radioactivity was still being excreted after the first day. Patient 32 who excreted 11.5 per cent of the injected tritium on the first day (secretory rate = 2,730 μg) excreted no detectable radioactive metabolite on the second day. Thus, in this single study, at least, the observed high secretion rate appears to be unrelated to renal failure, because all of the radioactive metabolite destined for excretion appeared within the first 24 hours. While this requirement has not actually been demonstrated in the other subjects with renal failure, the fact that these subjects often excreted about as much isotope as did the normal controls, and the fact that high secretion rates did not always correlate with renal failure, tend to indicate that the observed increases in aldosterone secretion are not related to kidney disease but rather to a truly increased adrenal production.

Because an abnormally prolonged excretion of isotopically labeled metabolite in renal disease would produce artificially high estimates of the 24 hour secretory rate, another group of ten patients has been studied in which the urinary collection periods were extended to two or three days, and the specific activity of labeled metabolite was determined for each of the days of collection. These data are summarized in Table III and in Figure 2. Included in this group are three pregnant women in the third trimester, four patients with renal diseases other than malignant hypertension, one patient with uncomplicated hypertension (normal renal function) and three patients with malignant hypertension.

Since accurate quantitation of the amount of radioactivity in the metabolite is difficult, its specific activity, derived from the ratio of H^3 to C^{14} in the triacetate of the metabolite, was determined instead. If the major portion of labeled metabolite is excreted in the first 24 hours, the H^3/C^{14} ratio for the first day should be much higher than that for subsequent days. The average secretion per day over a two or three day period was calculated from the average H^3/C^{14} ratio over the given period. When the average secretion rate was the same as the secretion rate determined for

TABLE III

The effect of delayed excretion of labeled tetrahydroaldosterone on the estimation of the aldosterone secretory rate

Patient and diagnosis	Renal status BUN	Renal status Proteinuria	Estimated secretory* rate (μg/day) Days	Estimated secretory* rate (μg/day) S. R.	Per cent of labeled metabolite accounted for	H^3/C^{14}† of aldosterone tetrahydro metabolite	Counts per minute‡ Released by glucuronidase	Counts per minute‡ In eluate of first chromatogram	Per cent of injected dose
1 (*a*) Lav. Primary hypertension	18	0	1	310	93.6	54.7	104,000	95,000	12
			2	290		1.7	21,000	1,500	0.16
			3				6,000	1,100	0.14
(*b*)	18	0	1	270	96.3	86.7	104,000	95,000	8.9
			2	260		4.3	44,000	7,000	0.65
			3	260		0.16	8,500	1,000	0.1
2 Ver. Pregnancy	12	+	1	750		9.5	50,000	35,000	12.5
			2	lost					
			3			0.3	2,500	3,000	1.0
3 Thom. Pregnancy	7	++	1	840	84.6	9.4	41,000	69,000	24.6
			2	710		1.6	7,700	23,000	8.2
4 Vict. Pregnancy	11	++++	1	1,200		6.6	30,000	43,000	15.3
			2				1,500		
5 (*a*) Du. Nephrosis	14	++++	1	380	89.5	22.8	33,000	22,000	5.5
			2	350	97.2	1.0	9,000	1,000	0.25
			3	340		0.36	5,000	500	0.1
(*b*)	14	++++	1	310	95.2	73.4	158,000	66,500	6.3
			2	295		3.7	37,000	3,000	0.3
			3	295		0.084	14,000	750	0.1
6 (*a*) Tayl. Malignant hypertension	35	+	1	925	89.8	9.7	43,000	16,000	4.1
			2	830		1.1	8,000	1,500	0.4
(*b*)	45	+	1	510	87.3	16.8	16,000	10,000	2.5
			2	445		2.4	5,500	4,500	1.1
7 Youn. Diabetic nephropathy	49	++	1	210	88.1	42		31,000	8.2
			2	185		6.2		5,000	1.4
			3				64,000	1,200	0.3
8 (*a*) Vel. Malignant hypertension	55	+++	1	740	77.0	11.6	23,500	18,000	4.6
			2	595	95.8	2.8	6,500	3,600	0.9
			3	570		0.5	1,000	1,000	
(*b*)	67	+++	1	665	73.7	13.5	30,000	13,000	3.3
			2	490		4.7	15,000	5,000	1.3
			3				5,000		
9 Kirt. Nephrosclerosis	89	+++	1	200		43.2	23,000	10,000	2.6
			2				13,000	2,200	0.6
10 (*a*) Chris. Sodium-losing nephritis	86	0	1	1,000		9.7	26,000	11,000	2.6
			2				14,000	6,200	1.5
			3			0.14	11,000	1,500	0.4
(*b*)	104	0	1	1,320	73.5	18.0	77,000	60,000	5.5
			2	970		6.4	25,000	20,000	1.8
11 Dunc. Malignant hypertension	185	++++	1	14,600		0.59	8,000	4,000	1.0
			2	7,500		0.54	10,000	3,000	0.75
			3	6,000		0.30	4,000	2,000	0.50

* The secretion rate (S.R.) for Days 2 and 3 is calculated from

$$\frac{H^3/C^{14}\text{ Day 1} + H^3/C^{14}\text{ Day 2} + \cdots H^3/C^{14}\text{ Day n}}{n}; \text{ and } \frac{(\text{S.R.})n}{n} = \text{S.R./day.}$$

Per cent of labeled metabolite accounted for is the per cent of completeness of excretion of the labeled metabolite of the

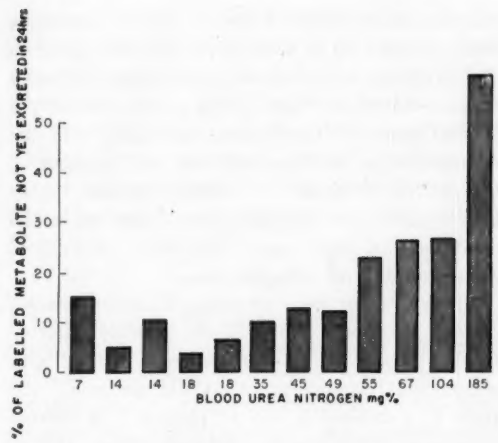

FIG. 2. RELATION OF UREA RETENTION TO RETENTION OF ALDOSTERONE METABOLITE AFTER 24 HOURS. Aldosterone secretion rates which are based on the collection of a single 24 hour urine collection are artificially high because of incomplete excretion of labeled tetrahydroaldosterone. The per cent of labeled metabolite destined for excretion which had not yet been excreted in the first 24 hours was determined in each instance by serial urine collections which allowed more complete excretion of the labeled metabolite. The blood urea nitrogen values are presented below each of the patients studied. It can be seen that in patients without urea retention the error caused by delayed excretion is small because about 90 per cent of the labeled metabolite has been excreted. The excretion rate of labeled metabolite appears to be inversely related to the level of the blood urea nitrogen. However, delayed excretion of metabolite does not appear to modify the 24 hour secretory rate determination to a significantly greater degree until urea retention is relatively advanced. The value obtained in the patient with a blood urea nitrogen of 185 mg per 100 ml was considered invalid since no evidence for complete excretion of labeled metabolite could be obtained (see text).

the first day, all of the labeled metabolite was excreted during the first 24 hours. As a general principle, the excretion of labeled metabolite can be assumed to be complete when the estimate of the average daily secretion rate is no longer modified by inclusion of subsequent urine collections in the determination. By dividing the average secretory rate (S.R. obtained over n days) by the S.R. based only upon the first 24 hour collection, the fraction of the total accounted for metabolite which was excreted within the first day can be estimated. This fraction $\times$ 100 = the percentage of labeled metabolite accounted for in the first day, and these percentages are given in column 6 of Table III. When the S.R. for Day 1 is multiplied by this fraction, a value for the S.R. is obtained which has been corrected for delayed isotope excretion. Further, $100 - \frac{\text{S.R. Day n}}{\text{S.R. Day 1}}$ yields the percentage of counts not yet excreted after the first 24 hours, and this latter value, therefore, also represents the percentage by which the first 24 hour collection is artificially high because of incomplete excretion (Figure 2).

Normally, about 90 per cent of the labeled metabolite is excreted in the first 24 hours. One might then expect that measurements of secretion rates in normal subjects might be falsely high by about 10 per cent. This expectation has actually been demonstrated in three subjects with normal blood urea nitrogen values (Table III, Patients 1, 3 and 5). In five different serial studies of these three subjects, determination of the secretion rate based on two or three day urine collections indicates that estimates based only on the first 24 hour collections were artificially high by from 3.7 to 15.4 per cent (96.3 to 84.6 per cent of labeled metabolite was excreted in the first 24 hours).

In the application of this method to the study of diseases associated with renal insufficiency, such as malignant hypertension, the critical question is whether delayed excretion of the isotope causes overestimation of the 24 hour secretory rate beyond the 10 per cent error normally encountered. The data presented in Table III indicate that renal insufficiency can, in fact, lead to secretion rate values which should be reduced by as much as

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first 24 hours = $\frac{\text{Avg. S.R. n days}}{\text{S.R. Day 1}} \times 100$. $\frac{\text{This value}}{100}$ multiplied by the observed secretory rate of the first 24 hours = the secretory value corrected for delayed excretion (C.S.R.).

† The H^3/C^{14} ratio is presented. The percentage change from Day 1 to Day 2 or to Day 3 may not necessarily correspond with the percentage change in the secretory rate because the specific activity of the C^{14}-acetic anhydride used for acetylation varied slightly in different batches.

‡ Counts per minute not corrected for quenching or promotion effects due to pigments in urinary extracts. The per cent of injected dose = $\frac{\text{no. of counts in metabolite zone of first chromat.}}{\text{no. of counts injected}} \times 100$.

26.5 per cent (or, more properly, by the 16.5 per cent value in excess of the normal error). However, in most instances, the error is less. The 26.5 per cent figure was obtained in a patient with a blood urea nitrogen of 104 mg per 100 ml (Patient 10). Thus, the rate of excretion of the labeled metabolite by two subjects with overt renal insufficiency (Patients 6 and 7) did not differ significantly from patients with normal renal function. Of special interest is the finding that in two patients with malignant hypertension and azotemia (Patients 6 and 8), both tested twice, the percentages of labeled metabolite excreted in the first day were 89.8, 87.3, 77 and 74 per cent.

One comatose patient with malignant hypertension and terminal uremia, who died the day after the last collection, has been included. This patient was obviously moribund. Unlike all others, the specific activity of the metabolite did not fall between the first and second days and fell by only 50 per cent in three days. The minimum estimated secretory rate was 6,000, but this result is obviously invalid because no evidence for completeness of excretion of metabolite could be obtained before death of the patient. The number of counts excreted was small and constituted a trace of the injected dose. In contrast, in the one patient of the present series of malignant hypertensive subjects with a similarly high estimated secretory rate of 10,000 μg (Patient 37, Table II) both the amount and the percentage of labeled metabolite excreted were similar to that of normal subjects and were considerably higher than those observed in Patient 11 of Table III. The possible error associated with this estimated 10,000 μg production value was not determined, but from the experience summarized in Table III it seems likely that the corrected value would be considerably elevated above the normal range.

The experience summarized in Table III and in Figure 2 illustrates that the renal excretion of labeled tetrahydroaldosterone is inversely related to the excretion of urea. The data indicate that a secretion rate value in patients with azotemia may need to be corrected by as much as about 30 per cent. The data suggest that a correction factor based on the degree of azotemia should be applied to each of the secretion rates given in Table I in order to obtain a more accurate secretory rate value. However, it appears from reexamination of Table I that even if a correction error of about 30 or even 40 per cent is applied to the malignant hypertensive patients studied, most of the abnormally high values remain above the normal limits. This finding, combined with the demonstration of hypersecretion in a number of the patients who did not exhibit nitrogen retention, suggests that the high values observed in this condition are not merely the result of delayed renal excretion of the metabolite.

Evidences for hypersecretion of other adrenal cortical hormones. Four of the patients reported in the present study were tested for their glucocorticoid response to an ACTH infusion. Patient 11 exhibited a rise in plasma 17-hydroxycorticoids (Porter-Silber method) from 31.2 to 51.6 μg per 100 ml after an ACTH infusion. Patient 15 responded by an increase in plasma hydroxycorticoids from 17.6 to 41.5 μg per 100 ml. (This test was done after one adrenal had been removed.) Patient 23 exhibited a rise in plasma 17-hydroxycorticoids of from 35.7 to 58.0 μg per 100 ml. These values are all considered to be within the upper limit of normal. Patient 27 has been previously reported in detail by Hilton, Westermann, Bergen and Crampton (15), and exhibited an abnormal response to the infusion of ACTH. This patient is the only patient in the series in whom hyper-responsiveness to ACTH was clearly present. In ten other patients of the present series the urinary 17-hydroxycorticosteroids and 17-ketogenic steroids were measured, and all of these values were within normal limits.

Correlation of findings with morphological changes in the adrenal tissue. In seven of the patients with malignant hypertension reported in this study, complete postmortem examination has been made. No tumor was found in the adrenals of any of these patients, but in four of the seven the adrenal glands were significantly above the upper limits of normal in weight. In all seven subjects the glands exhibited areas of focal hyperplasia and nodularity, especially in the region of the glomerulosa zone. In addition, there was focal increase in cellular activity and increased lipid deposits in focal cellular areas. Pathological findings in these cases, as well as in two other surgical specimens, will be the subject of a separate report (16).

Effect of various pharmacological agents. The

effect of various antihypertensive drugs on the secretion rate of aldosterone is unknown. Certain of the patients (10 of 33) included in this series were under treatment with various hypotensive agents, and this has been recorded in Table I. From the results obtained, these agents do not appear to have had any consistent or specific effect on the secretion rate of aldosterone.

DISCUSSION

Estimation of the adrenal production rate by isotope dilution as carried out in the present study possesses a number of inherent advantages over the previously employed methods which have measured the small fraction of hormone excreted unchanged in the urine. However, it should be recognized that while the measurement of adrenal secretory rate would seem to be a more precise and reliable index of aldosterone activity, it too, may not always be an accurate reflection of the effective concentration of active hormone in the blood. Possibly, blood level measurements may provide additional information in this regard, and ultimately may even be necessary to assure proper interpretation of secretion rate studies.

The data presented suggest that a well defined biochemical difference exists between a group of patients classified as benign essential hypertension and those who manifest the clinical picture of advanced or of malignant hypertensive disease. Marked increases in the secretion rate of aldosterone were found in all but one of the patients with malignant hypertension and in three of eight patients classified as "advanced" hypertension. These increased values were observed in the presence of normal sodium intakes. In contrast, all of the patients with primary hypertension secreted normal amounts of aldosterone.

Thus, malignant hypertension represents another syndrome which is associated with increased secretion of aldosterone. While this clinical state resembles the disease produced in animals given excess mineralocorticoid, it clearly differs from the clinical picture of primary aldosteronism in that evidences of vasculitis are characteristically absent in the latter condition.

The frequent occurrence of hypokalemic alkalosis as manifested by either an elevated plasma bicarbonate and/or a reduced plasma potassium concentration in patients with advanced or with malignant hypertension appears as a somewhat unexpected finding. This tendency to alkalosis may be taken as supportive evidence of increased aldosterone secretion. The alkalosis may have additional significance since it was found to occur at times in the presence of renal failure where retention of fixed anions ordinarily tends to lower the plasma bicarbonate level and thus could mask the typical plasma abnormalities of aldosteronism.

In the patients reported here hypersecretion of aldosterone was often found in association with demonstrable renal insufficiency. One cannot deny that sodium-losing renal disease could lead to secondary hypersecretion of aldosterone in patients with advancing hypertensive disease. However, as pointed out above, the data obtained thus far do not tend to suggest the operation of such a mechanism. (Most of the patients conserved sodium normally.) Further study of patients with other forms of renal disease may be necessary to determine a possible relationship between aldosterone secretion and renal insufficiency. It should be restated here that the technique employed for estimation of adrenal secretion may be affected by renal insufficiency, but the error caused by delayed excretion has not been of sufficient magnitude to invalidate the results.

In general, the hypersecretion of aldosterone was more impressive in the seriously ill patients (10 of the 15 patients with malignant hypertension are now deceased). However, whether the aldosterone hypersecretion is a cause or a result of the severe disease is not clear. Hypersecretion of aldosterone has not been noted in association with various other severe illnesses (17), but here too, further studies are necessary, especially in patients with other forms of arteritis.

Prior to the present study, the association of potassium depletion and alkalosis with severe hypertension has been noted. Wyngaarden, Keitel and Isselbacher (18) reported the case of a 46 year old man with malignant hypertension and severe alkalosis whose adrenal glands were grossly normal at postmortem. Two other cases of potassium depletion associated with malignant hypertension and high aldosterone excretion have been reported (19, 20). In both, the authors considered that the observed aldosteronism might have been secondary to renal damage. Both of these

patients succumbed without being helped by bilateral adrenalectomy, and here too, the adrenal glands were not grossly abnormal.

About 35 cases of primary aldosteronism have now been reported (17). About 28 of these have been shown to be associated with an adrenal adenoma, and two others were associated with adrenal carcinoma (21, 22). Hypertension of varying degree was present in all of the cases, but in only one of the cases associated with an adenoma was the hypertension malignant (23). This patient died without operation. In marked contrast, and of special interest in relation to the present study, is the fact that in five of the six reported cases (15, 24–28) of primary aldosteronism associated with bilateral adrenal cortical hyperplasia, the syndrome of malignant hypertension with papilledema was present. In all but one of these six patients subtotal adrenalectomy was carried out and was followed by improvement. The fact that the syndrome of malignant hypertension was so often found in these patients labeled "primary aldosteronism due to bilateral adrenal hyperplasia," illustrates again a fundamental difficulty in properly classifying these diseases. Obviously, these reported cases might well have been more appropriately classified as examples of "malignant hypertension" rather than of "primary aldosteronism."

The finding of an adrenal adenoma at least suggests a primary adrenal disease. On the other hand, the occurrence of bilateral adrenal hyperplasia is in keeping with secondary rather than with primary adrenal disease. In other words, the two diseases may be of entirely different origin. Also, they appear to be associated with different clinical pictures—bilateral adrenal hyperfunction being characteristically associated with malignant hypertension. However, as yet, no biochemical differences are apparent which might aid one in distinguishing the disease state of bilateral hyperplasia from that of an adrenal adenoma. Four of the cases of bilateral hyperplasia occurred at a young age (ages 9, 10, 13 and 17) and one other case (15) exhibited an abnormal glucocorticoid response to ACTH. One of our patients (no. 20) resembles the patient reported by Holten and Petersen (25) in that unilateral adrenalectomy in both was followed by a fall in aldosterone and striking clinical improvement. In both patients the excised adrenal gland was histologically unremarkable. However, it is also possible that aldosterone hypersecretion remits spontaneously, and one of our patients (no. 34), a seven year old girl with malignant hypertension, has improved considerably on a medical regimen.

It is difficult to determine whether the clinical picture of malignant hypertension is caused by the excessive secretion of aldosterone or, alternatively, whether the hypersecretion is in some way a secondary reaction to the diseased state. That hypersecretion is a cause of the syndrome is suggested by the fact that the vascular lesions of malignant hypertension resemble those produced in animals given desoxycorticosterone (1). Moreover, the excessive production of aldosterone in these patients appears to be independent of the dietary sodium intake. On the other hand, certain observations suggest the contrary idea, that the hypersecretion is secondary to the underlying hypertensive disease. Thus, it is noteworthy that hypersecretion is absent in uncomplicated hypertension and becomes more and more prominent in association with development of renal and vascular complications. Secondly, the fact that the clinical picture is not the same as that of primary aldosteronism due to an adenoma suggests that pre-existing vascular disease is necessary for aldosterone hypersecretion to be associated with the malignant syndrome. This concept has support in animal studies wherein production of renal damage or a unilateral nephrectomy facilitates the production of the state of malignant hypertension by steroid administration.

In summary, the data appear to indicate that aldosterone hypersecretion does not participate in the pathogenesis of primary hypertensive disease. However, hypersecretion of aldosterone, perhaps superimposed on various hypertensive states, cannot be excluded as a causal factor in the development of the malignant syndrome. Viewed in this light, aldosterone hypersecretion may follow the hypertensive process but may possibly precede the syndrome of malignant hypertension. The nature of this stimulus to hypersecretion is unknown, but might be related to renal damage, because, regardless of the nature of the underlying hypertensive process, renal vascular damage is a most consistent finding in the malignant syndrome.

If aldosterone is important in the pathogenesis of malignant hypertension, one might ask whether beneficial effects are to be expected from bilateral adrenalectomy. Unfortunately, this question cannot be categorically answered since results of adrenalectomy have been variable, and since many patients with malignant hypertension have been excluded from surgery because of attendant renal insufficiency. One patient in our series (no. 25) was subjected to bilateral adrenalectomy, but following the second operation terminal uremia developed. Two similar experiences have been reported (19, 20). It is possible that a more satisfactory answer can be obtained when patients are operated on earlier in the malignant phase. However, in this regard it should be remembered that in animals, after a certain stage, hypertension progresses when desoxycorticosterone is no longer administered ("metacorticoid" hypertension) (29). Another, perhaps more promising, approach to the problem involves the administration of aldosterone inhibitors. These studies are currently under way in our laboratory.

The findings reported here perhaps confirm in part the work of Genest and associates (8) who reported increased urinary excretion of aldosterone in some patients with malignant hypertension. However, these workers also found increased aldosterone excretion in milder forms of hypertension, and this latter finding is at variance with our data.

SUMMARY

A technique of isotope dilution which involves the injection of a trace dose of tritiated aldosterone and the determination of the specific activity of labeled tetrahydroaldosterone in the subsequent 24 hour urine has been applied to the study of patients with hypertensive vascular disease. The technique obviates a difficulty inherent in previous studies which have been based on measurement of the small fraction of aldosterone excreted unchanged in urine.

In eight patients with benign essential hypertension, the adrenal secretion rate of aldosterone was found to be within the normal limits (180 to 330 μg per day). The values were similar to those obtained in eight normal subjects maintained on comparable normal intakes of sodium ion.

The secretion rate of aldosterone was also within the normal range in two patients with hypertension associated with unilateral renal disease.

Five patients with proven primary aldosteronism due to adrenal adenoma (Conn's syndrome) exhibited significantly increased secretory rates of from 510 to 1,690 μg per day.

In three of eight advanced hypertensive patients studied, and in all but one of 15 patients with malignant hypertension, the adrenal secretion rate of aldosterone was significantly elevated. The abnormal values ranged upwards from 510 μg per day in these patients. The patients with malignant hypertension resembled those with primary aldosteronism in that the hypersecretion was not associated with grossly reduced urinary sodium excretion. With unexpected frequency, the patients with malignant hypertension also exhibited a tendency to hypokalemia and/or an elevated plasma bicarbonate level, even in the presence of renal failure.

Delayed excretion of the labeled metabolite can be related to the degree of nitrogen retention present and may lead to artificially high estimates of the 24 hour secretion rate. Nevertheless, in the experience to date the error produced by excretory failure does not appear to be of sufficient magnitude to invalidate results based on 24-hour urine collections.

The results provide no indication that aldosterone hypersecretion participates in the pathogenesis of primary (benign) hypertension.

The data obtained on the patients studied establish that the syndrome of malignant hypertension is usually associated with hypersecretion of aldosterone. In addition to the differences in the clinical picture between patients with malignant hypertension and those with Conn's syndrome, the hypersecretion of aldosterone may differ in its origin. In malignant hypertension it seems to be due to bilateral adrenal hyperfunction rather than to an adrenal adenoma.

Hypersecretion of aldosterone in the syndrome of malignant hypertension may prove to be a secondary or concomitant phenomenon. However, the results presented here make it reasonable to consider the possibility that hypersecretion of aldosterone plays a causal role in malignant hypertension.

REFERENCES

1. Selye, H., Hall, C. E., and Rowley, E. M. Malignant hypertension produced by treatment with desoxycorticosterone acetate and sodium chloride. Canad. med. Ass. J. 1943, **49**, 88.
2. Perera, G. A., Knowlton, A. I., Lowell, A., and Loeb, R. F. Effect of desoxycorticosterone acetate on the blood pressure of man. J. Amer. med Ass. 1944, **125**, 1030.
3. Knowlton, A. I., Loeb, E. N., Stoerk, H. C., and Seegal, B. C. Development of hypertension and nephritis in normal and adrenalectomized rats treated with cortisone. Proc. Soc. exp. Biol. (N. Y.) 1949, **72**, 722.
4. Simpson, S. A., Tait, J. F., Wettstein, A., Neher, R., Von Euw, J., and Reichstein, T. Isolieurung eines neuen kristallisierten Hormons aus Nebennieren mit besonders hoher Wirksamkeit auf den Mineralstoff-Wechsel. Experientia (Basel) 1953, **9**, 333.
5. Conn, J. W. Primary aldosteronism, a new clinical syndrome. J. Lab. clin. Med. 1955, **45**, 3.
6. Ferrebee, J. W., Parker, D., Carnes, W. H., Gerity, M. K., Atchley, D. W., and Loeb, R. F. Certain effects of desoxycorticosterone; the development of "diabetes insipidus" and the replacement of muscle potassium by sodium in normal dogs. Amer. J. Physiol. 1941, **135**, 230.
7. Tobian, L., Jr., and Binion, J. I. Tissue cations and water in arterial hypertension. Circulation 1952, **5**, 754.
8. Genest, J., Koiw, E., Nowaczynski, W., and Leboeuf, G. Further studies on urinary aldosterone in human arterial hypertension. Proc. Soc. exp. Biol. N. Y. 1958, **97**, 676.
9. Ulick, S., Laragh, J. H., and Lieberman, S. The isolation of a urinary metabolite of aldosterone and its use to measure the rate of secretion of aldosterone by the adrenal cortex of man. Trans. Ass. Amer. Phycns 1958, **71**, 225.
10. Laragh, J. H. The effect of potassium chloride on hyponatremia. J. clin. Invest. 1954, **33**, 807.
11. Wilzbach, K. E. Tritium-labeling by exposure of organic compounds to tritium gas. J. Amer. chem. Soc. 1957, **79**, 1013.
12. Okita, G. I., Kabara, J. J., Richardson, F., and LeRoy, G. V. Assaying compounds containing H^3 and C^{14}. Nucleonics 1957, **15**, 111.
13. Stanbury, S. W., Gowenlock, A. H., and Mahler, R. F. Interrelationships of potassium deficiency and renal disease *in* Aldosterone. An International Symposium, A. F. Muller and C. M. O'Connor, Eds. Boston, Little, Brown & Co., 1958, pp. 155–166.
14. Rifkin, H., Solomon, S., and Lieberman, S. Role of the adrenal cortex in diabetic retinopathy and nephropathy. Diabetes 1958, **7**, 9.
15. Hilton, J G., Westermann, C. D., Bergen, S. S., and Crampton, R. S. Syndrome of mineralocorticoid excess due to bilateral adrenocortical hyperplasia. New Engl. J. Med. 1959, **260**, 202.
16. Lucas, F., and Laragh, J. H. To be published.
17. Laragh, J. H. Aldosterone in fluid and electrolyte disorders: Hyper and hypoaldosteronism. J. chron. Dis. 1960, **11**, 292.
18. Wyngaarden, J. B., Keitel, H. G., and Isselbacher, K. J. Potassium depletion and alkalosis, their association with hypertension and renal insufficiency. New Engl. J. Med. 1954, **250**, 597.
19. FitzGerald, M. G., Fourman, P., James, A. H., and Scarborough, H. Malignant hypertension, adrenal hyperplasia and depletion of potassium. Scot. Med. J. 1957, **2**, 473.
20. Eales, L., and Linder, G. C. Potassium losing pyelonephritis and malignant hypertension: A case report with balance studies. Metabolism 1959, **8**, 445.
21. Foye, L. V., Jr., and Feichtmeir, T. V. Adrenal cortical carcinoma producing solely mineralocorticoid effect. Amer. J. Med. 1955, **19**, 966.
22. Brooks, R. V., McSwiney, R. R., Prunty, F. T., and Wood, F. J. Potassium deficiency of renal and adrenal origin. Amer. J. Med. 1957, **23**, 391.
23. Flynn, P. Adrenal cortical tumour with fulminating hypertension. J. Irish med. Ass. 1956, **39**, 77.
24. van Buchem, F. S. P., Doorenbos, H., and Elings, H. S. Primary aldosteronism due to adrenocortical hyperplasia. Lancet 1956, **2**, 335.
25. Holten, C., and Petersen, V. P. Malignant hypertension with increased secretion of aldosterone and depletion of potassium. Lancet 1956, **2**, 918.
26. Bartter, F. C., and Biglieri, E. G. Primary aldosteronism: Clinical Staff Conference at National Institutes of Health. Ann. intern. Med. 1958, **48**, 647.
27. Kretchmer, N., Dickinson, A., and Karl, R. Aldosteronism in nine-year-old child (abstract). J. Dis. Child. 1957, **94**, 452.
28. Mellinger, R., Therian, B., Kline, I. T., Ditzler, J., Smith, R., and Fine, G. Primary aldosteronism. Henry Ford Hosp. Bull. 1958, **6**, 1.
29. Friedman, S. M., Friedman, C. L., and Nakashima, M. Sustained hypertension following the administration of desoxycorticosterone acetate. J. exp. Med. 1951, **93**, 361.

FACTORS INFLUENCING RELATIVE RATES OF SYNTHESIS OF ADULT AND FETAL HEMOGLOBIN *IN VITRO* *

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What causes the gradual decrease of fetal hemoglobin in the normal maturing infant, or the persistence of fetal hemoglobin in the blood of adults with certain congenital anemias, especially thalassemia, is not known. The production of fetal hemoglobin is evidently determined by genes nonallelic with those regulating adult hemoglobin formation (1). The fact that all red cells can be sickled in sickle cell anemia patients having relatively high proportions of fetal hemoglobin indicates that the same cell can produce both an adult hemoglobin (i.e., S) and fetal hemoglobin. This in turn implies that the rate of formation of fetal hemoglobin is not dependent upon the formation of a different cell by a different gene, but reflects a metabolic adaptation by the individual young red cells of the fetus or of the abnormal adult to the cellular environment (2).

The present investigation concerns the effect of cellular environment on the relative quantities of fetal and adult hemoglobin synthesized *in vitro* by the reticulocytes of umbilical cord blood, as determined by the rates of Fe^{59} incorporation into heme and of C^{14}-leucine incorporation into globin.

MATERIALS AND METHODS

Fe^{59} was obtained from Abbott Laboratories, and iron-binding protein of high degree of purity was a gift from Dr. John K. Inman (3). To aqueous solutions of the protein, buffered with 20 mM sodium bicarbonate so as to yield a final pH of 7.4 ± 0.2, sufficient iron either as $Fe^{59}Cl_3$ or $Fe^{56}Cl_3$, was added dropwise to one-third saturate the iron-binding protein. To insure complete protein-binding of the added iron, the mixture was allowed to stand at room temperature for 1 hour prior to its experimental use.

Leucine uniformly labeled with C^{14} was purchased from Nuclear of Chicago and had a specific activity of 43 μc per mg. Nonradioactive amino acids were obtained from Schwarz Bioresearch Laboratories. IRC-50 resin was supplied by Rohm and Haas Company and purified and equilibrated as previously described (4).

Incubation. From 30 to 50 ml of sterile heparinized umbilical cord blood was obtained at the time of delivery. It was centrifuged at 2,000 G for 30 minutes at 0° C and the top 5 ml of red cells, relatively rich in reticulocytes, was withdrawn and washed twice with Krebs-Ringer phosphate with glucose solution, pH 7.4, at 0° C. In the studies of glucose deficiency, glucose was omitted. An approximately 50 per cent suspension of the washed red cells was made with the same solution and a hematocrit value and reticulocyte count obtained. Two ml aliquots of this red cell suspension were then pipetted into 25 ml siliconized Erlenmeyer flasks. To this was added 1 ml of the amino acid mixtures in Krebs-Ringer phosphate with glucose solution so that their final concentration was twice that of normal fasting levels, as determined by Stein and Moore (5), with the exception of glycine which was made up to 4 times its normal value. In certain experiments valine, isoleucine and histidine were omitted. In other experiments, in which C^{14}-leucine was used as a tracer, the radioactive amino acid was substituted for the stable form.

The red cell suspension was preincubated in a Dubnoff metabolic incubator at 37° C with shaking at approximately 20 times per minute. In experiments on the effects of hypoxia this was done in separate tents under water-saturated gas mixtures containing 95 per cent O_2 and 5 per cent CO_2 or 95 per cent N and 5 per cent CO_2 at a flow rate of 1 L per minute. In other experiments the samples were incubated under water-saturated room air. After 1 hour, there was added sufficient Fe^{59}-labeled iron-binding protein to yield a final concentration of 24 μM and sufficient C^{14}-leucine to yield a final concentration of 2.6 mg per 100 ml. To alternate flasks equal amounts of Fe^{56}-containing iron-binding protein and C^{14}-leucine were added. The flasks were then incubated 4 hours longer.

Separation of hemoglobins. The period of incubation was terminated by the addition of 10 volumes of 0.85 per cent NaCl at 0° C (6) and the red cells were washed 5 times in 0.85 per cent NaCl at 0° C, suspended in 1 ml of 0.85 per cent NaCl and dialyzed overnight against 250 ml of distilled water. The resulting hemolysate was removed from the dialysis sack, 0.8 ml of toluene was added, the solution was shaken vigorously for 5 minutes, after

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which 0.4 ml of 5 per cent NaCl was added. The solution was then centrifuged at 2,000 G for 30 minutes or until the middle hemoglobin-containing layer was clear. The hemoglobin was removed by pipetting and dialyzed for 24 hours against a phosphate buffer, 0.06 M in sodium ions, 0.01 M in cyanide ions, pH 6.91 ± 0.02 [Developer no. 4 (4)]. After removal from the dialysis sack, the hemoglobin solutions were found to have a concentration of 70 ± 10 mg per ml. One ml of each sample was then pipetted on a 0.9 × 35 cm column of IRC-50 resin, previously equilibrated against Developer no. 4 and the sample was washed into the column with 0.2 ml samples of developer. The columns were then developed for 5 to 7 hours at 4° C with Developer no. 4 flowing at a rate of 5 to 7 ml per hour (4). By then the main component of adult hemoglobin was 1 to 5 cm from the top of the column, and F_{II}, the main component of fetal hemoglobin (4), was approximately 12 to 30 cm from the top of the column. The resin was removed by extrusion and the individual bands were dissected out and eluted from the resin with 3 ml of saturated disodium phosphate at room temperature. The pH of this eluted hemoglobin solution was 7. The hemoglobin concentration in each band was determined at 540 mμ on a Coleman Junior spectrophotometer. The fetal hemoglobin concentration obtained from the F_{II} band is expressed as the percentage of the total eluted hemoglobin. Since this main component of F hemoglobin includes about 80 per cent of the total fetal hemoglobin (4), the values will be approximately 20 per cent lower than one might expect from alkali denaturation.

Determination of radioactivity. Hemoglobin labeled with Fe^{59} was counted in a well-type scintillation counter. In four experiments, after addition of known amounts of nonradioactive hemoglobin as carrier, the heme was also separated from the globin and crystallized to constant specific activity after the method of Shemin (7). In all instances the value of the activity per milligram of hematin chloride corresponded to that of hemoglobin, assuming heme to constitute 3.9 per cent of hemoglobin. Thus the activity is reported in terms of hemoglobin for ease in comparison with C^{14}-leucine labeling of globin.

In experiments in which C^{14}-leucine was used, the hemoglobin, after elution from the column, was dialyzed against 0.001 M NaCN, the concentration of the hemoglobin was determined, and 0.1 ml samples were pipetted on stainless steel planchets. These samples were then counted with a windowless flow counter, and corrected to a sample size of 0.5 mg using an empirical self-absorption curve. Experiments on large samples demonstrated that all the radioactivity of C^{14}-leucine-labeled hemoglobin, after it had been chromatographically purified, was in the globin fraction and was precipitable by 5 per cent trichloroacetic acid.

RESULTS

Effect of hypoxia. Table I shows the results of six experiments done to investigate the effects of hypoxia on the relative amounts of adult hemoglobin and of fetal hemoglobin synthesized. Since the ratio of fetal/adult hemoglobin is variable from one cord blood sample to another, only comparison with the control ratio is informative. In the last column is given the percentage change in the ratio produced by hypoxia. In every case there

TABLE I

Effect of hypoxia on the relative rates of synthesis of adult and fetal hemoglobin by cord blood reticulocytes in vitro

Label	Reticulocytes	F_{II}*	95% O_2			95% N_2			Per cent increase Ratio $\frac{FN_2/AN_2 - FO_2/AO_2}{FO_2/AO_2}$
			FO_2	AO_2	Ratio FO_2/AO_2	FN_2	AN_2	Ratio FN_2/AN_2	
	%	%	*cpm/mg Hb*			*cpm/mg Hb*			
Fe^{59}	4.9	57	25	120	0.208	27	97	0.278	34
Fe^{59}	1.1	60	70	201	0.348	130	315	0.413	19
Fe^{59}	6.7	75	43	123	0.350	111	168	0.661	89
			35	87	0.402	66	113	0.584	45
Fe^{59}	15.2	50	1,129	2,270	0.497	613	1,034	0.593	19
			1,117	2,311	0.483	633	1,004	0.630	30
As heme†:									
2nd crystallization			1,424	2,668	0.534	763	1,180	0.647	21
3rd crystallization			1,586	2,947	0.538	858	1,228	0.699	30
Fe^{59}	8.0	59	1,094	2,208	0.495	671	1,142	0.588	19
C^{14}-leucine			1,396	2,630	0.531	1,048	1,785	0.587	11
Fe^{59}	6.0	68	408	866	0.471	863	1,520	0.568	21
C^{14}-leucine			883	1,728	0.511	1,172	2,168	0.541	6

* Main component of fetal hemoglobin determined by chromatography.
† Counted as crystallized heme, but specific activity calculated as hemoglobin.

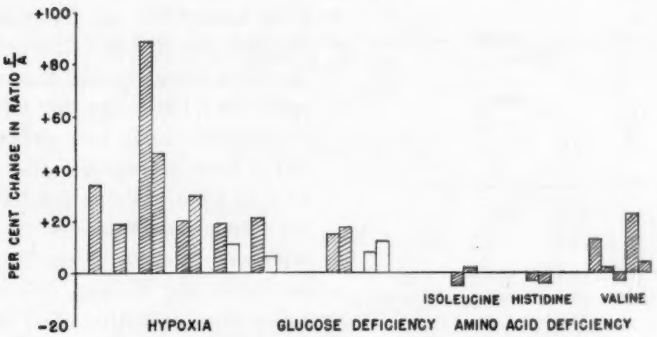

FIG. 1. EFFECT OF VARIOUS DEFICIENCIES ON THE RELATIVE SYNTHESIS OF FETAL AND ADULT HEMOGLOBIN BY UMBILICAL CORD RETICULOCYTES *IN VITRO*. The percentage change in the ratio $\frac{\text{fetal hemoglobin synthesized}}{\text{adult hemoglobin synthesized}}$ as compared to a nondeficient control is shown. Bar graphs for experiments in which Fe^{59} was used as a label are shaded, while those for C^{14}-leucine experiments are unshaded.

was a rise in the ratio of radioactive fetal/adult hemoglobin, indicating that hypoxia produced a relative stimulation of synthesis of fetal hemoglobin compared to adult hemoglobin. A chi-square test of the ten incubations done here shows significance at the 0.5 per cent level. Figure 1 summarizes the effects of hypoxia, as well as other factors, on relative fetal hemoglobin production.

Although hypoxia uniformly stimulated fetal hemoglobin synthesis relative to adult hemoglobin synthesis, Table I demonstrates a variable effect of oxygen deprivation on the specific activity of hemoglobin (A and F combined). Red blood cells represent a peculiarly difficult tissue in which to obtain complete anoxia because of the large reservoir of oxygen in the hemoglobin. It was supposed that the variable effect of hypoxia on the specific activity might be a result of different degrees of oxygen deprivation achieved, since there is evidence that with complete anoxia no hemoglobin synthesis would occur (8). Hence, in two similar experiments, one of which is illustrated in Figure 2, we departed from the usual protocol. The Erlenmeyer flasks were stoppered with airtight vaccine caps and preincubated for 1.5 hours with the following gas mixtures: 95 per cent O_2 and 5 per cent CO_2; 13 per cent O_2, 82 per cent N_2 and 5 per cent CO_2; and 95 per cent N_2 and 5 per cent CO_2. In one flask incubated with 95 per cent N_2 and 5 per cent CO_2, a well containing 0.5 ml of 35 per cent hydrosulfite was employed to further decrease the oxygen tension. The Fe^{59}-iron-binding protein was then added under nitrogen, and the sealed flasks incubated for 5.5 hours longer. The percentage saturation of each hemoglobin sample was then determined by the method of Gordy and Drabkin (9). The treatment of the samples and the crystallization of the heme to constant specific activity were then carried out as usual. It can be seen from Figure 2 that mild hypoxia tended to increase the hemoglobin specific activity. This increase in specific activity may not reflect increased hemoglobin synthesis since, for example, hypoxia may cause a shrinkage of the intracellular pool of nonlabeled iron during preincubation, with a consequent lesser dilution of the radioactive label. The consistently greater relative stimulation of fetal hemoglobin synthesis under conditions of severe hypoxia is evident again.

An attempt was made to determine the time course of the effects of hypoxia on fetal and adult hemoglobin synthesis. Equal portions of the reticulocyte-rich umbilical cord red cells were incubated in four separate Erlenmeyer flasks with Fe^{59}-labeled iron-binding protein as usual, two under 95 per cent O_2 and 5 per cent CO_2, and two under 95 per cent N_2 and 5 per cent CO_2. After two hours the incubation was stopped for a pair of flasks, one oxygenated and one hypoxic, while the remaining pair was incubated for four hours as usual. The result of this experiment is shown

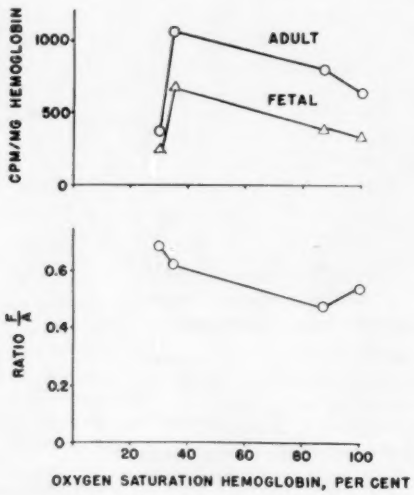

FIG. 2. EFFECT OF VARIOUS DEGREES OF OXYGEN DEFICIENCY ON THE SYNTHESIS OF ADULT AND FETAL HEMOGLOBIN *IN VITRO*. The changes in specific activity of the adult and fetal hemoglobins recovered are depicted in the top portion of the figure; the ratio of fetal hemoglobin specific activity to adult hemoglobin specific activity is shown in the bottom portion.

in Figure 3. A significant change in the ratio of hemoglobin synthesized did not occur until the last two hours of the four hour incubation. This was due apparently to a relatively greater decrease in the rate of fetal hemoglobin production in the 95 per cent O_2 environment.

Effect of metabolic inhibition. The effect of glucose deficiency, cyanide and methylene blue may be seen in Table II. Glucose deficiency, like oxygen lack, depressed adult hemoglobin synthesis relatively more than fetal hemoglobin synthesis. The addition of 120 mg per 100 ml desoxyglucose to the glucose-deficient medium had no additional effect. Both cyanide and methylene blue almost completely stopped synthesis of both hemoglobins, so that the ratios are of little quantitative significance, but are included to demonstrate that inhibition of these types did not favor fetal hemoglobin synthesis.

Effect of amino acid deficiencies. The effect of various amino acid deficiencies may be seen in Table III. Fetal and adult human hemoglobin differ in valine, isoleucine, and histidine content. Fetal hemoglobin has less valine than adult hemoglobin has (9.5 vs 11.2 g per 100 g), the missing valine being replaced almost mole for mole by isoleucine (1.46 g per 100 ml), which the main component of adult hemoglobin lacks entirely (4, 10). Fetal hemoglobin also has less histidine (7.7 vs 8.4 g histidine per 100 g) (10). Thus, the effects of deficiencies of these three amino acids were studied. From Table III we see that isoleucine, and histidine deficiencies in the incubation medium had no effect on Fe uptake. Valine deficiency in two experiments seemed to increase relative fetal hemoglobin synthesis and although the results here are not significant, they suggest further study.

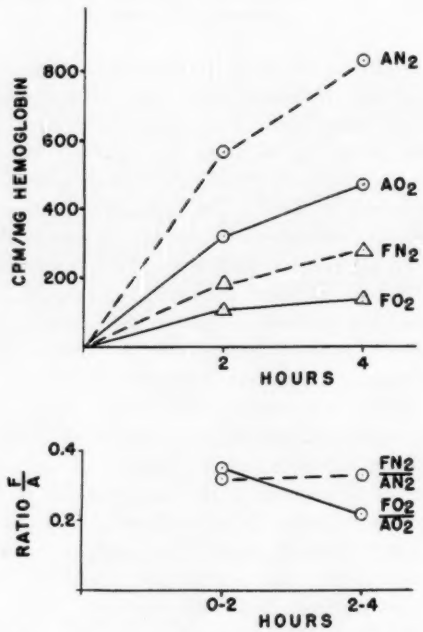

FIG. 3. TIME COURSE OF SYNTHESIS OF FETAL AND ADULT HEMOGLOBIN BY UMBILICAL CORD RETICULOCYTES UNDER NITROGEN AND UNDER OXYGEN *IN VITRO*. The rate of synthesis of both A hemoglobin (⊙) and F hemoglobin (△) diminished during the second two-hour period of incubation both under oxygen (——) and under nitrogen (– – –). Diminution in hemoglobin synthesis was greatest in respect to the rate of F hemoglobin synthesis under oxygen. In the lower diagram the ratio of fetal to adult hemoglobin synthesized is compared for the first two-hour period with the ratio for the second two-hour period.

TABLE II

Effect of glucose deficiency, cyanide, and methylene blue on the relative synthesis of adult and fetal hemoglobin by cord blood reticulocytes in vitro

Label	Reticulocytes	F_{II}*		Addition to flask	F	A	Ratio F/A	Per cent change from control ratio F/A in inhibited flask
	%	%			*cpm/mg Hb*			
Fe^{59}	2.3	56	Control	Glucose	137	332	0.41	
			Inhibited	0	149	319	0.47	15
					138	290	0.48	17
C^{14}-leucine	4.5	66	Control	Glucose	603	1,154	0.52	
					577	1,160	0.50	
			Inhibited	120 mg % desoxyglucose	646	1,154	0.56	8
					645	1,157	0.56	12
			Inhibited	Glucose + 10^{-2} NaCN	31	91	0.34	−33
					24	91	0.26	−49
			Inhibited	Glucose + 0.002% methylene blue	28	86	0.33	−35
					35	109	0.32	−37

* Main component of fetal hemoglobin determined by chromatography.

DISCUSSION

Hypoxia uniformly favored the production of fetal hemoglobin compared with adult hemoglobin as seen in Table I and Figure 1. In the presentation of these experiments, we have emphasized the *ratio* of fetal and adult hemoglobin synthesized rather than their individual rates of synthesis. Since both fetal and adult hemoglobin-synthesizing mechanisms apparently share the same pool, this ratio of the specific activity of two hemoglobins, made in the same cell, is presumably not influenced by changes in the intracellular pool size. Although, as mentioned earlier, both F and A hemoglobin are probably synthesized within the same cells, there is some evidence that the proportions of adult and fetal hemoglobins may vary somewhat from one cell to another (11). Thus it is possible that the adult and fetal synthesizing mechanisms are present in young red cells in varying amounts. This should not, however, invalidate the measurement of the ratio of hemoglobins synthesized.

The generally accepted explanation of the function of fetal hemoglobin is that it represents a specific adaptation for life *in utero*, by providing the fetus with red cells with a greater affinity for oxygen (12). While human fetal red cells do indeed have a greater affinity for oxygen at a given Po_2 than do adult red cells (i.e., the oxygen dissociation curve is "shifted to the left") (12, 13), the curve for hemolyzed fetal red cells is actually to the right of that of a hemolysate of maternal red cells, and when both hemoglobins have been dialyzed their oxygen dissociation curves become identical (14). Furthermore, whereas fetal red cells may lose their heightened oxygen affinity within a month after birth (12), fetal hemoglobin

TABLE III

Effect of deficiency of various amino acids on rates of adult and fetal hemoblogin synthesis in vitro by cord blood reticulocytes, using Fe^{59} incorporation into hemoglobin

Reticulocytes	F_{II}*	Complete			−Isoleucine			Per cent change $\frac{F_I/A_I - F_c/A_c}{F_c/A_c}$	Deficient −Histidine			Per cent change $\frac{F_I/A_I - F_c/A_c}{F_c/A_c}$	−Valine			Per cent change $\frac{F_I/A_I - F_c/A_c}{F_c/A_c}$
		F_c	A_c	Ratio	F_I	A_I	Ratio F_I/A_I		F_I	A_I	Ratio F_I/A_I		F_I	A_I	Ratio F_I/A_I	
%	%	*cpm/mg Hb*			*cpm/mg Hb*				*cpm/mg Hb*				*cpm/mg Hb*			
5.6	46	147	369	0.40	144	380	0.38	−5					105	233	0.45	13
4.3	61	196	410	0.48	182	375	0.49	2					146	300	0.49	2
4.6	74	120	346	0.35					115	340	0.34	−3	110	321	0.34	−3
2.4	66	61	129	0.47					59	131	0.45	−4	46	80	0.58	23
													40	82	0.49	4

* Main component of fetal hemoglobin determined by chromatography.

disappears at a more leisurely pace (15). Thus, the concept that the primary function of fetal hemoglobin is for its specific properties in oxygen transport is difficult to defend. However, the possibility that the increased affinity of fetal hemoglobin for oxygen within the red cell is due to a property of fetal hemoglobin not evident in solution, has not been ruled out.

An alternate explanation is suggested by the consideration that the organism producing fetal hemoglobin has many other demands for oxygen, energy-yielding metabolites, and essential amino acids. Hence, a protein with a smaller content of certain essential amino acids and requiring less energy to produce might possess an advantage for the fetus.

The importance of hypoxia, glucose deficiency, or possibly valine deficiency in directly regulating fetal hemoglobin production can only be suggested, not determined, by these *in vitro* experiments. At birth the blood flowing to the fetus from the placenta, before respiration, has usually been found to be about 50 per cent saturated with oxygen (16). Certainly one might expect this degree of saturation to be further reduced in the tissue capillaries. The fetus seems to be well supplied with glucose, although the possibility of local deficiency cannot be excluded. Valine deficiency is evidently not severe in the fetus (17) but local tissue deficiency may exist. Although the changes in synthesis rates reported here are small, it must be remembered that the incubation period was carried out during only a small segment of the total time of hemoglobin synthesis by the maturing red cell. Therefore, an effect of one or all of these deficiencies in the direct regulation of fetal hemoglobin production seems possible.

The observation that time of birth during fetal life has no discernible effect on the rate of replacement of fetal hemoglobin (18, 19) is against any direct mediation of oxygen on the regulation of fetal hemoglobin production. Since rapid growth rate, rather than intrauterine existence, might condition local glucose or valine deficiency, this same observation is less opposed to the idea of these substances as regulators.

Regardless of the role of these deficiencies in the regulation of fetal hemoglobin production in the normal infant, it is possible that fetal hemoglobin is an adaptation to these deficiencies. This possibility provides impetus for the investigation of defects of energy or amino acid metabolism in congenital anemias of adults characterized by large amounts of fetal hemoglobn, such as thalassemia.

SUMMARY

1. Reticulocyte-rich suspensions of umbilical cord blood red cells were incubated *in vitro* with Fe^{59}-iron-binding protein, or C^{14}-leucine, and the amounts of Fe^{59}-labeled or C^{14}-labeled fetal or adult hemoglobin newly synthesized were measured separately.

2. Conditions of hypoxia, or glucose deficiency, favored the formation of fetal hemoglobin as compared to adult hemoglobin.

3. It is possible that fetal hemoglobin may be an adaptation to the fetal environment in which oxygen and glucose are at a premium.

ADDENDUM

Since this paper was submitted for publication a communication presenting similar observations has appeared [Thomas, E. D., Lochte, H. L., Jr., Greenough, W. B., II., and Wales, M. *In vitro* synthesis of foetal and adult haemoglobin in foetal haematopoietic tissues. Nature (Lond.) 1960, **185**, 396]. This finding of an increase in the proportion of hemoglobin F synthesis relative to hemoglobin A with anoxia, is in agreement with our results despite differences in tissue used, length of incubation, radioactive label, and method of isolation of the hemoglobins.

REFERENCES

1. White, J. C., and Beaven, G. H. Foetal haemoglobin. Brit. Med. Bull. 1959, **15**, 33.
2. Itano, H. A. The human hemoglobins: Their properties and genetic control. Advanc. Protein Chem. 1957, **12**, 215.
3. Inman, J. K. Preparation of crystalline beta$_1$-metal combining protein *in* Proceedings of the Tenth Conference on the Plasma Proteins and Cellular Elements of the Blood, sponsored by the Protein Foundation, Inc. Cambridge, Mass., 1956, p. 46.
4. Allen, D. W., Schroeder, W. A., and Balog, J. Observations on the chromatographic heterogeneity of normal adult and fetal human hemoglobin: A study of the effects of crystallization and chromatography on the heterogeneity and isoleucine content. J. Amer. chem. Soc. 1958, **80**, 1628.
5. Stein, W. H., and Moore, S. The free amino acids of human blood plasma. J. biol. Chem. 1954, **211**, 915.

6. Allen, D. W., and Jandl, J. H. Kinetics of intracellular iron in rabbit reticulocytes. Blood 1960, **15**, 71.
7. Shemin, D. Biosynthesis of protoporphyrin *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press Inc., 1957, vol. 4, p. 643.
8. Thomas, E. D. *In vitro* studies of erythropoiesis. II. The effect of anoxia on heme synthesis. Blood 1955, **10**, 612.
9. Gordy, E., and Drabkin, D. L. Spectrophotometric studies. XVI. Determination of the oxygen saturation of blood by a simplified technique, applicable to standard equipment. J. biol. Chem. 1957, **227**, 285.
10. Stein, W. H. Observations of the amino acid composition of human hemoglobins *in* Conference on Hemoglobin. Washington, National Academy of Sciences, National Research Council, 1958, p. 220.
11. Singer, K., and Fisher, B. Studies on abnormal hemoglobins. V. The distribution of type S (sickle cell) hemoglobin and Type F (alkali resistant) hemoglobin within the red cell population in sickle cell anemia. Blood 1952, **7**, 1216.
12. Darling, R. C., Smith, C. A., Asmussen, E., and Cohen, F. M. Some properties of human fetal and maternal blood. J. clin. Invest. 1941, **20**, 739.
13. McCarthy, E. F. The oxygen affinity of human maternal and foetal haemoglobin. J. Physiol. (Lond.) 1943–44, **102**, 55.
14. Allen, D. W., Wyman, J., Jr., and Smith, C. A. The oxygen equilibrium of fetal and adult human hemoglobin. J. biol. Chem. 1953, **203**, 81.
15. Jonxis, J. H. P. Foetal haemoglobin and Rh antagonisms *in* Haemoglobin-Barcroft Symposium. New York, Interscience Publishers, Inc., 1949, p. 261.
16. Smith, C. A. The Physiology of the Newborn Infant, 2nd ed. Springfield, Ill., Charles C Thomas, 1951.
17. Allen, D. W. The concentrative ability of normal adult, fetal, and thalassemic human reticulocytes for different amino acids. In preparation.
18. Walker, J., and Turnbull, E. P. N. Haemoglobin and red cells in the human foetus. III. Foetal and adult haemoglobin. Arch. Dis. Childh. 1955, **30**, 111.
19. Cook, C. D., Brodie, H. R., and Allen, D. W. Measurement of fetal hemoglobin in newborn infants. Pediatrics 1957, **20**, 272.

METABOLISM OF MUCOPOLYSACCHARIDES IN CONNECTIVE TISSUE. II. SYNTHESIS OF GLUCOSAMINE-6-PHOSPHATE *

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New methods of studying metabolic processes in connective tissue provide opportunity to obtain information concerning enzymatic steps in the synthesis of mucopolysaccharides in that tissue. These techniques supplement studies done in microorganisms and in other tissues (1–3), and allow investigation of phenomena of interest in relation to the diseases of connective tissue at an enzymatic level. Glucosamine and galactosamine are present in the disaccharide repeating units of the mucopolysaccharides of connective tissue. Uridine diphosphoacetylglucosamine has been shown to be the donor of acetylglucosamine in hyaluronate synthesis and the precursor of uridine diphosphoacetylgalactosamine which serves a similar function in chondroitin sulfate synthesis (1). An initial step in the synthesis of uridine diphosphoacetylglucosamine is the synthesis of glucosamine-6-phosphate.

The present report concerns the activity of the enzyme involved in the synthesis of glucosamine-6-phosphate in connective tissue and liver of rats and in human synovial tissues, as well as investigation of the influence of cortisol and gold compounds on the activity of this enzyme. A comparison of the effects of cortisol on several aspects of connective tissue metabolism has also been made.

METHODS

Polyvinyl (Ivalon) sponges were inserted under the dorsal skin of male Wistar albino rats weighing 200 ± 50 g, anesthetized with pentobarbital (50 mg per kg). The animals were sacrificed 2 weeks later; the sponges and liver were removed, weighed and homogenized separately. Human synovial tissue obtained at the time of arthrotomy for orthopedic surgery was homogenized within a few minutes after removal.

Fructose-6-phosphate-glutamine transamidase. The activity of this enzyme was determined by a modification of the procedure of Pogell and Gryder (4). Homogenates were made in 0.15 M KCl, with 0.001 M disodium ethylenediamine tetraacetate (EDTA), 0.065 per cent mercaptoethanol, and 0.024 M glutamine, pH 7.0. Assays were done in a volume of 2 ml containing 20 μmoles of fructose-6-phosphate, 25 μmoles of glutamine and 50 μmoles of phosphate buffer, pH 7.7. After 15 to 60 minutes at 37° C, the incubation mixture was heated at 100° for 2 minutes, centrifuged, and the hexosamine content of an aliquot of the supernatant solution was determined following conversion to acetylhexosamine by the procedure of Roseman and Daffner (5), using the method of Reissig, Strominger and Leloir (6). Glucosamine, subjected to the same procedure, was used as reference standard. The absorption spectrum of the product of the enzymatic reaction matched that of the reference standard.

Glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase. Activity of these enzymes was determined using homogenates made in 0.1 M phosphate buffer, pH 7.5. Glutamic-oxaloacetic transaminase was determined using 0.1 mmole of aspartate, 0.02 mmole of α-ketoglutarate and 0.2 mmole of phosphate buffer, pH 7.5, in a total volume of 2.0 ml. After 20 minutes at 37° C, the solutions were heat inactivated at 100° for 2 minutes, centrifuged, and 0.2 ml of supernatant was then added to 0.2 mg reduced diphosphopyridine nucleotide (DPNH), 400 units of malic dehydrogenase (Sigma), and 60 mmoles of phosphate buffer, pH 7.5, in a total volume of 2.0 ml. After incubation for 20 minutes, DPNH disappearance was determined fluorometrically, following the procedure of Lowry, Roberts and Kapphahn (7). Glutamic-pyruvic transaminase was assayed in a similar fashion, using 50 μmoles of alanine and 400 units of lactic dehydrogenase (Sigma) in place of the aspartate and malic dehydrogenase. The amount of homogenate used in each assay was kept within the range which would give zero order reaction and a straight line relationship between the amount of homogenate and the amount of DPNH oxidized. Protein determinations were done by ultraviolet absorption (8).

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† John and Mary R. Markle Scholar in Medical Sciences.

RESULTS

Activity of the transamidase which synthesizes glucosamine-6-phosphate from fructose-6-phosphate and glutamine was demonstrated in liver and in the connective tissue which entered polyvinyl sponges in rats, and in human synovial tissue samples. Expressed as micromoles of hexosamine formed per milligram of protein in the crude homogenate, connective tissue homogenates usually had a slightly greater activity than did liver homogenates from each animal (Table IV). The findings in human synovial tissue are given in Table I. No correlation was found between the level of enzymatic activity and the diagnosis, age of the patient or histological appearance of the tissue in a limited group of individuals studied. The lowest value was found in a man with ankylosing spondylitis who was noted to have marked softening of the femoral head at the time of surgery.

TABLE I

Fructose-6-phosphate-glutamine transamidase activity of human synovial tissue obtained surgically

Site	Age	Sex	Diagnosis	Glucosamine synthesis
				μmoles $\times 10^{-4}$/*hr/mg protein*
Knee	67	M	Traumatic arthritis with effusion	97
Knee	46	M	Osteochondritis desiccans	61
Hip	40	F	Aseptic necrosis of femoral head	56
Elbow	58	M	Traumatic bursitis	49
Tendon	33	F	Ganglion	47
Knee	31	M	Fractured patella	42
Knee	58	M	Fractured patella	37
Knee	30	M	Fractured patella	22
Elbow	37	M	Traumatic bursitis	22
Knee	54	F	Traumatic bursitis	19
Hip	25	F	Aseptic necrosis of femoral head	14
Hip	56	M	Ankylosing spondylitis	6

Synthesis of hexosamine occurred in connective tissue homogenates with either fructose-6-phosphate or glucose-6-phosphate, but little or no synthesis occurred when other sugars were substituted (Table II). Hexosamine synthesis occurred in liver homogenates with a variety of sugars, although the greatest activity was found with fructose-6-phosphate or mannose-6-phosphate. Ammonium chloride and glutamine were compared as donors of the amino groups in both systems; these results were summarized in Table III.

TABLE II

*Comparison of the synthesis of hexosamine by connective tissue homogenates with equimolar amounts of various substrates**

Substrate	Homogenate used	
	Connective tissue	Liver
Fructose-6-phosphate	100	100
Glucose-6-phosphate	99	97
Galactose-6-phosphate	12	70
Mannose-6-phosphate	15	106
Fructose	0	71
Glucose	0	48
Galactose	0	68
Mannose	0	56

* Figures are percentage of synthesis with fructose-6-phosphate.

Effect of cortisol (Table IV). Beginning the seventh day after the insertion of the polyvinyl sponges, rats were injected daily with 5 mg of cortisol subcutaneously for one week before sacrifice. Cortisol administration caused a decrease in the weight and protein content of the granuloma. A fall in fructose-6-phosphate-glutamine transamidase activity occurred, but this change was not statistically significant. The glutamic-oxaloacetic transaminase activity in the connective tissue rose to a significant extent, but there was no change in the glutamic-pyruvic transaminase activity.

The fall in fructose-6-phosphate-glutamine transamidase activity in the liver was of the same magnitude as that in connective tissue. This dosage of cortisol produced very definite increases in the hepatic glutamic-oxaloacetic and glutamic-pyruvic transaminase activities. Cortisol was not found to influence the activity of these enzymes

TABLE III

Assay of relative effectiveness of glutamine and NH_4Cl as donors of amine group in glucosamine-6-phosphate synthesis

Donor added	Glucosamine synthesis (*μmoles* $\times 10^{-4}$/*hr/mg protein*) Homogenate used	
	Connective tissue	Liver
Glutamine, 12.5 *mmoles*	55.6	23.8
NH_4Cl, 12.5 *mmoles*	17.2	15.3
Neither	11.8	19.8

TABLE IV

*Effect of cortisol on sponge connective tissue and liver of rats**

		Connective tissue			Liver		
		Mean	SEM†	No.	Mean	SEM†	No.
Weight of granuloma (g)	Treated	1.48	± 0.07	12			
	Control	1.93	± 0.133	17			
		p = < 0.01					
Protein content of granuloma (*mg*)	Treated	142	±26.9	12			
	Control	297	±41.4	17			
		p = < 0.01					
Fructose-6-phosphate-glutamine transamidase	Treated	29.4	± 6.74	12	21.6	±5.55	11
	Control	41.9	± 5.03	17	32.4	±4.16	17
		p = < 0.1			p = < 0.1		
Glutamic-oxaloacetic transaminase	Treated	9.54	± 1.35	11	73.9	±6.50	12
	Control	6.45	± 0.83	14	44.5	±4.95	14
		p = < 0.05			p = < 0.01		
Glutamic-pyruvic transaminase	Treated	9.47	± 1.45	11	15.0	±1.92	12
	Control	8.88	± 1.13	14	5.1	±0.80	14
		p = < 0.3			p = < 0.001		

* Data for enzyme activities are in the following units: fructose-6-phosphate transamidase, μmoles × 10^{-4}/hr/mg protein; glutamic-oxaloacetic transaminase, μmoles/hr/mg protein; glutamic-pyruvic transaminase, μmoles/hr/mg protein for liver and μmoles × 10^{-2}/hr/mg protein for connective tissue.

† Standard error of the mean.

in connective tissue or in liver homogenates when added *in vitro*.

Effect of gold chloride in vitro. Since EDTA and mercaptoethanol were necessary to protect the fructose-6-phosphate-glutamine transamidase in tissue homogenates, it seemed possible that gold salts might influence the activity of the enzyme. Homogenates made in medium containing EDTA, but no mercaptoethanol, were incubated with and without a series of potential inhibitors. Gold chloride inhibited the activity of this enzyme in connective tissue homogenates in a lower concentration than in liver homogenates (Table V). Other sulfhydryl reactants such as iodoacetamide, iodobenzoic and *p*-chloromecuribenzoate were also found to inhibit the activity of this enzyme in connective tissue homogenates, although the concentration of these substances needed to give 50 per cent inhibition was ten times that found for gold chloride.

The effect of gold sodium thiomalate in vivo. One week after insertion of sponges, 10 mg of gold sodium thiomalate (Myochrysine) was administered subcutaneously. Seven to ten days later enzyme assays were performed. A marked decrease in the activity of the fructose-6-phosphate-glutamine transamidase was found in the connective tissue homogenates, but no effect was noted on the activity of the enzyme in liver homogenates obtained from the same animals (Table VI).

The acid mucopolysaccharide concentration of the sponge connective tissue in three of the animals injected with gold sodium thiomalate was de-

TABLE V

Effect of gold chloride and other inhibitors on the activity of the fructose-6-phosphate-glutamine transamidase in rat tissue homogenates

		Per cent inhibition	
	Molarity (× 10^{-4})	Connective tissue	Liver
Gold chloride	500	100	100
	50	100	89
	5	84	27
	3.75	38	
	2.5	24	5
	1.25	16	6
	0.5	0	
Iodoacetamide	500	100	81
	50	56	9
	37.5	48	
	25	33	
	12.5	7	12
	5	0	6
	2.5		0
Iodobenzoate	50	54	
	25	31	
	5	6	
p-Chloromercuribenzoate	50	52	
	25	42	
	5	26	

TABLE VI

*Effect of gold sodium thiomalate administration on fructose-6-phosphate-glutamine transamidase in rats**

	Connective tissue			Liver		
	Mean	SEM†	No.	Mean	SEM†	No.
Treated	12.9	±3.04	11	31.5	±3.48	11
Control	41.9	±5.03	17	32.4	±4.16	17
	$p = < 0.001$					

* Activity is expressed as μmoles × 10^{-4}/hr/mg protein.
† Standard error of the mean.

termined by a method previously described (9). Expressed as uronic acid content of the acid mucopolysaccharide fraction, a decrease from an average of 85 mg per 100 g of dry connective tissue in control animals to 52 mg per 100 g in the treated animals was noted.

DISCUSSION

Many of the characteristics of the enzyme involved in the synthesis of glucosamine-6-phosphate were demonstrated comparatively easily in homogenates of the granulation tissue which formed in polyvinyl sponges. Similar homogenates of liver tissue contained a lower activity of the fructose-6-phosphate-glutamine transamidase and a relatively greater concentration of other enzymes which interfered with the characterization of the substrates of the transamidase.

Cortisone and cortisol have been shown to inhibit connective tissue growth, fibril formation, sulfate fixation and oxygen consumption (10). An objective of this study was to determine if adrenal steroids uniformly inhibit all metabolic activities of connective tissue, or whether selective effects on certain metabolic functions occur. The glutamic-pyruvic and glutamic-oxaloacetic transaminases were studied in connective tissue since an increase in the activities of these enzymes in the liver of rats given cortisol has been reported (11, 12). Our finding of an increase in glutamic-oxaloacetic transaminase activity in connective tissue after cortisol administration indicates that this agent does not uniformly inhibit all metabolic activities in that tissue. Cortisol and gold sodium thiomalate were found to have different effects on liver than on connective tissue metabolism; the former increased glutamic-pyruvic transaminase activity only in the liver, while the latter depressed fructose-6-phosphate-glutamine transamidase activity only in connective tissue. These findings indicate the need for direct study of connective tissue metabolism.

It has been assumed that gold compounds exert their effects on patients with rheumatoid arthritis through inhibition of enzymes with labile sulfhydryl groups (13). Evidence has been obtained in animals that the effects of gold compounds used therapeutically are due to the gold content of these agents and not to the rest of the molecule, regardless of the type of linkage involved (14, 15). The need to protect the enzyme which synthesizes glucosamine-6-phosphate from sulfhydryl inhibitors *in vitro* led to assay of gold compounds on the activity of this enzyme. Concentrations of gold which caused inhibition in connective tissue homogenates were of the order of magnitude found in the serum of patients receiving gold compounds for therapy of rheumatoid arthritis (16).

SUMMARY

The transamidase, which synthesizes glucosamine-6-phosphate, and the glutamic-oxaloacetic and glutamic-pyruvic transaminases were studied in connective tissue and liver of rats, and in human synovial tissue. Cortisol was found to cause a moderate diminution in the activity of the fructose-6-phosphate-glutamine transamidase, but caused an increase in the glutamic-oxaloacetic transaminase activity of connective tissue homogenates. No change in the glutamic-pyruvic transaminase activity occurred. In the liver, cortisol administration caused a moderate decrease in fructose-6-phosphate-glutamine transamidase activity and marked increase in glutamic-oxaloacetic and glutamic-pyruvic transaminase activities. Gold chloride inhibited the activity of the transamidase which synthesizes glucosamine-6-phosphate in connective tissue homogenates *in vitro* in concentrations which are found in the serum of patients receiving gold compounds therapeutically. Gold sodium thiomalate administration *in vivo* resulted in decrease in the activity of this enzyme in connective tissue but not in liver.

REFERENCES

1. Roseman, S. Metabolism of connective tissue *in* Annual Review of Biochemistry. Palo Alto, Annual Reviews, Inc., 1959, vol. 28, p. 545.
2. Bollet, A. J., Goodwin, J. F., and Brown, A. K. Metabolism of mucopolysaccharides in connective tissue. I. Studies of enzymes involved in glucuronide metabolism. J. clin. Invest. 1959, **38**, 451.
3. Kizer, D. E., and McCoy, T. A. The synthesis of hexosamine in tumor homogenates. Cancer Res. 1959, **19**, 307.
4. Pogell, B. M., and Gryder, R. M. Enzymatic synthesis of glucosamine-6-phosphate in rat liver. J. biol. Chem. 1957, **228**, 701.
5. Roseman, S., and Daffner, I. Colorimetric method for determination of glucosamine and galactosamine. Analyt. Chem. 1956, **28**, 1743.
6. Reissig, J. L., Strominger, J., and Leloir, L. F. A modified colorimetric method for the estimation of N-acetylamino sugars. J. biol. Chem. 1955, **217**, 959.
7. Lowry, O. H., Roberts, N. R., and Kapphahn, J. I. The fluorimetric measurement of pyridine nucleotides. J. biol. Chem. 1957, **224**, 1047.
8. Layne, E. Spectrophotometric and turbidimetric methods for measuring proteins *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press Inc., 1955, vol. III, p. 447.
9. Bollet, A. J. The measurement of tissue acid mucopolysaccharides. J. clin. Invest. 1958, **37**, 858.
10. Taubenhaus, M. Hormonal influences on granulation tissue formation *in* The Healing of Wounds, Martin B. Williamson, Ed. New York, McGraw-Hill, 1957, p. 113.
11. Rosen, F., Roberts, N. R., Budnick, L. E., and Nichol, C. A. An enzymatic basis for the gluconeogenic action of hydrocortisone. Science 1958, **127**, 287.
12. Rosen, F., Roberts, N. R., and Nichol, C. A. Glucocortico-steroids and transamidase activity. I. Increased activity of glutamic-pyruvic transaminase in four conditions associated with gluconeogenesis. J. biol. Chem. 1959, **234**, 476.
13. Freyberg, R. H., Block, W. D., and Levey, S. Metabolism, toxicity and manner of action of gold compounds used in the treatment of arthritis. J. clin. Invest. 1951, **20**, 401.
14. Preston, W. S., Block, W. D., and Freyberg, R. H. Chemotherapy of progressive arthritis of mice. I. Role of sulfur in gold-containing compounds. Proc. Soc. exp. Biol. (N. Y.) 1942, **50**, 253.
15. Denko, C., and Anderson, A. K. Studies on the toxicity of gold compounds in rats. J. Lab. clin. Med. 1944, **29**, 1168.
16. Goodwin, J. F., and Bollet, A. J. The microdetermination of gold in the serum and urine of patients receiving chrysotherapy. J. Lab. clin. Med. In press.

THE EFFECT OF ESTROGENS UPON THE PERIPHERAL METABOLISM OF THYROXINE * †

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In plasma, thyroxine is avidly bound to specific proteins present in low concentration, particularly the so-called thyroxine-binding globulin (TBG) and thyroxine-binding pre-albumin (TBPA) (1–4). Several observations indicate that such protein-hormone interactions are of physiological importance. *In vitro,* the ability of cellular systems to accumulate thyroxine from suspending media is conditioned by the thyroxine-binding potency of the proteins within the media (5, 6). In addition, the volume of distribution and rate of turnover of thyroxine *in vivo* resemble those of certain plasma proteins (7, 8). Finally, abnormal thyroxine-binding by TBG and TBPA has been observed in a variety of states in which the peripheral metabolism of thyroid hormone is disturbed (9–12). It therefore seemed possible that interactions with plasma proteins might in part regulate the peripheral utilization and degradation of thyroxine.

An ideal test of this hypothesis would be provided by studies of hormonal metabolism performed before and during the administration of large quantities of purified binding protein. However, adequate quantities of these materials are not yet available. Therefore, it has been necessary to assess the effects of alterations in thyroxine-binding induced by pharmacological means. Large doses of estrogen have been shown to induce pronounced increases in the thyroxine-binding activity of TBG (13), with little or no change in the binding activity of TBPA (14). The present studies concern the effects of estrogens and of the increase in thyroxine-binding by TBG which they induce on the peripheral metabolism of thyroxine in patients with diverse states of thyroidal function.

MATERIAL AND METHODS

By means of techniques described in detail elsewhere (7), the distribution and peripheral turnover of I^{131}-labeled thyroxine [1] have been assessed before and during maximum estrogen-induced increase in circulating TBG. Definitions of terms employed and their mode of calculation are noted in the appendix. Patients studied included 8 women with treated myxedema, 3 with intact thyroidal function, and 4 with thyrotoxic Graves' disease. In 10 of the eumetabolic patients, paired studies were performed before and during the administration of diethylstilbestrol. Comparison of the effects of diethylstilbestrol and estradiol benzoate were made in 5 of these patients.

The sequence of the study periods, details of thyroidal replacement therapy, and dosage and duration of estrogenic therapy in each subject are presented in Table I. Duration of estrogenic therapy ranged between 19 and 54 days, and measurements of thyroxine turnover during periods of estrogenic therapy were performed during the last 8 to 10 days of the treatment period. Subjects with intact thyroidal function, excepting V.D., were given 30 mg of methimazole by mouth every 6 hours during each study period in order to prevent thyroidal recycling of I^{131} liberated by peripheral deiodination of radioactive thyroxine. The serum protein-bound iodine (PBI) [2] and thyroxine-binding activity of TBG were assessed at 3 day intervals throughout control and treatment periods.

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[1] Obtained from Abbott Laboratories, Oak Ridge, Tenn.

[2] Determined by Bio-Science Laboratories, Beverly Hills, Calif.

TABLE I

*Effect of estrogen on several parameters of thyroidal economy**

Subject	Study period	Therapy: Thyroidal replacement†	Therapy: Total duration of therapy‡	TDS	$T_{1/2}$	k	C	PBI	ETT	D	TBG§: Range	TBG§: Mean	BMR‖
		mg/day	*days*	*L*	*days*	*%/day*	*L/day*	*μg %*	*μg I/day*	*μg I/day*			%
Treated myxedema													
1	Control	180		7.6	6.0	11.6	0.88	9.2	699	81.1	48.4–54.9	51.9	−4
	Diethylstilbestrol	180	35	7.7	7.2	9.6	0.74	10.2	785	75.4	70.6–74.0	72.3	+1
2	Control	0.2¶		9.4	6.9	10.2	0.96	5.7	536	54.7	60.5–61.2	60.8	0
	Diethylstilbestrol	0.2	26	10.5	10.4	6.7	0.70	9.5	998	66.9	78.2–82.0	81.1	+6
3	Control	180		8.2	6.6	10.5	0.86	5.0	410	43.0	52.2–54.3	53.5	+5
	Diethylstilbestrol	180	35	8.8	8.4	8.3	0.73	7.5	660	54.8	81.7–84.2	82.6	−3
4	Control	240		8.9	5.5	12.6	1.12	5.1	454	57.2	47.1–54.8	50.9	+12
	Diethylstilbestrol	240	42	9.0	7.6	9.1	0.82	6.9	621	56.5	72.8–80.6	77.2	+7
5	Control	240		9.8	6.7	10.3	1.01	6.7	657	67.7	59.5–63.5	61.5	+12
	Diethylstilbestrol	240	33	10.0	7.8	8.9	0.89	8.1	810	72.1	81.8–90.5	86.2	+18
	Estradiol benzoate	240	28	10.1	7.0	9.9	1.00	8.1	818	81.0	65.0–71.2	68.9	+7
6	Control	180		10.4	6.8	10.2	1.06	3.8	395	40.3	46.4–48.5	48.3	0
	Diethylstilbestrol	180	24	10.5	9.5	7.3	0.77	4.5	472	34.3	80.2–82.4	81.3	−4
	Estradiol benzoate	180	25	10.5	8.8	7.9	0.83	5.9	620	49.0	73.7–77.0	75.0	+6
7	Control	180		9.1	7.6	9.1	0.83	4.0	364	33.1	50.8–53.0	51.9	−8
	Diethylstilbestrol	180	38	8.3	10.5	6.6	0.55	4.6	382	25.2	87.4–90.9	89.2	+2
	Estradiol benzoate	180	38	8.8	8.8	7.9	0.70	4.4	387	30.6	80.8–90.1	86.8	−4
8	Control	180		13.9	7.0	9.9	1.38	4.2	584	57.8	45.2–54.0	50.2	−7
	Estradiol benzoate	180	54	14.4	8.5	8.2	1.18	5.6	806	66.1	66.7–78.0	70.6	−8

* For explanation of abbreviations, see Appendix.

† Thyroid extract, USP.

‡ Estrogenic treatment, either 30 mg diethylstilbestrol daily by mouth or 11.0 mg estradiol benzoate 3 times weekly intramuscularly, or where indicated, propylthiouracil alone.

§ Per cent radioactivity bound to TBG at a concentration of added thyroxine of 0.475 μg/ml.

‖ Mean of 4 values obtained during study period.

¶ Sodium L-thyroxine pentahydrate.

TABLE I—*Continued*

Subject	Study period	Therapy: Thyroidal replacement†	Therapy: Total duration of therapy‡	TDS	$T_{1/2}$	k	C	PBI	ETT	D	TBG§ Range	TBG§ Mean	BMR‖
		mg/day	*days*	*L*	*days*	*%/day*	*L/day*	*μg %*	*μg I/day*	*μg I/day*			*%*
Intact thyroid function													
9	Control			7.8	6.5	10.7	0.84	5.5	429	45.9	44.2–46.5	45.5	+6
	Diethylstilbestrol		19	9.1	11.0	6.3	0.57	7.9	719	45.3	64.5–78.3	72.6	+11
10	Control			9.1	5.8	11.9	1.08	6.3	573	68.2	48.2–50.4	49.3	+8
	Diethylstilbestrol		43	10.1	7.9	8.8	0.89	7.5	758	66.7	69.8–76.1	73.0	+14
	Estradiol benzoate		45	8.5	8.3	8.3	0.71	7.5	638	53.0	59.4–71.6	65.5	+10
11	Control			7.8	7.2	9.6	0.75	6.9	538	51.6	58.0–59.6	61.0	+12
	Diethylstilbestrol		28	8.8	9.0	7.7	0.68	8.9	783	60.3	72.7–75.1	73.9	+5
	Estradiol benzoate		28	7.3	9.7	7.1	0.52	8.5	620	44.0	72.5–86.4	79.5	+2
Hyperthyroidism													
12	Diethylstilbestrol		25	9.5	4.8	14.4	1.37	10.5	998	143.7	62.6–65.4	66.6	+10
	Untreated			9.5	3.4	20.4	1.94	11.2	1,064	217.1	44.0–51.2	49.4	+34
	Propylthiouracil		29	9.4	4.4	15.8	1.48	6.0	564	89.1	57.0–65.1	61.1	+12
13	Diethylstilbestrol		22	10.0	6.4	10.8	1.08	10.3	1,030	111.2	77.1–81.0	79.0	+7
	Untreated			10.1	4.2	16.5	1.67	9.9	1,000	165.0	60.0–60.4	60.2	+30
	Propylthiouracil		33	10.2	5.3	13.1	1.34	8.2	836	109.5	57.7–67.1	62.4	+16
14	Diethylstilbestrol		40	10.1	8.7	8.0	0.81	11.8	1,192	95.4	81.9–91.4	86.6	+23
	Untreated			9.7	7.7	9.0	0.87	10.2	989	89.0	55.0–59.3	57.1	+35
	Propylthiouracil		47	9.5	6.5	10.7	1.02	5.1	484	51.8	64.2–71.6	68.0	+4
15	Diethylstilbestrol		44	8.6	4.0	17.3	1.49	17.1	1,471	254.5	64.2–71.1	68.0	+58
	Untreated			8.4	3.6	19.2	1.61	14.0	1,176	225.8	56.8–59.0	57.9	+60
	Propylthiouracil		88	8.6	4.0	17.3	1.49	7.5	645	111.6	40.6–45.5	43.0	+11

TABLE II

*Effect of estrogens on the fecal excretion and degradation of thyroxine**

Patient	F_{max}		Fecal clearance		Degradative clearance		Fecal excretion		Degradative rate	
	Control	Estrogen	Control	Estrogen	Control	Estrogen	Control	Estrogen	Control	Estrogen
	% dose		*L/day*		*L/day*		*μg I/day*		*μg I/day*	
1	27.5	38.9	0.24	0.29	0.64	0.45	22.4	29.3	58.7	46.1
2	37.4	40.6	0.36	0.29	0.60	0.42	20.5	27.2	34.2	39.7
3	29.8	28.7	0.26	0.21	0.60	0.52	12.8	15.8	39.7	30.2
4	42.0	29.5	0.47	0.24	0.65	0.58	24.0	16.6	33.1	39.9
5	23.2	16.2	0.23	0.14	0.78	0.75	15.7	11.7	51.9	60.4
8	21.6	9.6	0.30	0.11	1.08	1.07	12.5	6.3	45.3	59.8
10	26.7	26.2	0.29	0.23	0.79	0.66	18.2	17.5	50.0	49.2
Mean	29.7	27.1	0.31	0.22	0.74	0.63	18.0	18.1	43.3	47.7
Mean diff.	−2.6		−0.09		−0.10		+0.1		+4.4	
SEMD†	3.3		−0.035		−0.027		2.2		3.3	
p	>0.4		<0.05		<0.01		>0.5		>0.2	

* For definition of terms, see Appendix.
† Standard error of the mean difference.

Studies of thyroxine turnover during the treatment period were begun when a new steady state had been achieved, as indicated by constancy of values for TBG and PBI. Values for TBG presented in Table I represent the range and mean of 4 to 5 measurements made during control or steady state treatment periods.

Additional studies were performed during periods in which the PBI and the binding activity of TBG were changing after the initiation or withdrawal of estrogenic hormone. In Patient 6, the fractional rate of peripheral turnover of thyroxine was observed during a 24 day period beginning immediately after institution of treatment with diethylstilbestrol. Patient 9 was studied from the fifth through the eighteenth day following cessation of therapy with diethylstilbestrol.

The 4 women with thyrotoxicosis were studied while hospitalized on a metabolic ward. Diethylstilbestrol was administered only after constancy of basal oxygen consumption had been achieved. After attainment of maximal augmentation in TBG during the administration of estrogen, studies were again performed. Thereafter, all therapy was discontinued until TBG levels, concentrations of PBI and TBG, and basal oxygen consumption stabilized at values comparable to those found prior to estrogenic treatment. Thyroxine turnover studies were then repeated. At the termination of these tests, the patients were given propylthiouracil in dosage and duration sufficient to induce clinical and laboratory evidences of eumetabolism. Studies of peripheral turnover of thyroxine were then repeated.

Estimates of the thyroxine-binding activity of TBG were obtained by means of a technique, described in detail elsewhere (15), which has since been superseded in the authors' laboratories (4). In brief, the method employed consisted of conventional paper electrophoresis in veronal buffer, pH 8.6, of serum labeled with I^{131}-thyroxine and enriched to several standard concentrations with stable thyroxine. At each concentration, the percentage distribution of added hormone between TBG and albumin was assessed in both experimental sera and concomitantly electrophoresed control sera. As indicated in an earlier communication (15) alterations in the binding activity of TBG are associated with qualitatively similar changes in the distribution of thyroxine between TBG and albumin at each of the concentrations of hormone employed. Therefore, only values obtained at a single concentration, 47.5 μg thyroxine per 100 ml, are presented. This technique does not afford an estimate of the absolute thyroxine-binding capacity of TBG. Nevertheless, comparison of changes in TBG which occur during pregnancy, assessed by this method (15) and by methods which permit estimation of binding maxima (16), indicate that the two techniques provide qualitatively similar information.[3]

Statistical analyses were performed by the "paired *t*" test, as described by Snedecor (17). An earlier study suggested that the presence of a functioning thyroid gland does not alter the kinetics of thyroxine turnover peripherally, since values for normal subjects and patients with treated myxedema were similar (7). Therefore, in the present studies, results related to the effects of estrogen on the peripheral turnover of thyroxine in all eumetabolic normal and athyrotic subjects were pooled.

RESULTS

Data obtained in individual patients are presented in Tables I and II. Mean values and statistical intercomparisons are summarized in Tables II and III.

[3] Neither estimates of the thyroxine-binding activity of TBG, as herein obtained, nor of its absolute binding capacity afford information concerning the absolute quantity of protein present. Presumably, alterations in binding activity may reflect changes in either the concentration of TBG or its binding stoichiometry.

TABLE III

*Summary of the effects of estrogen on thyroidal economy**

	Study period	Duration of estrogenic therapy	TDS	$T_{1/2}$	k	C	PBI	ETT	D	TBG†	BMR
			L	*days*	*%/day*	*L/day*	*μg %*	*μg I*	*μg I/day*		*%*
Eumetabolic subjects given diethylstilbestrol (n = 10)											
Mean	Control		8.8	6.6	10.7	0.94	5.8	506	54.3	53.5	+4
Mean	Diethylstilbestrol	32	9.3	8.9	7.9	0.73	7.6	699	55.8	79.5	+6
SEMD‡			0.2	0.3	0.3	0.03	0.3	40	2.4	2.3	2
p			<0.05	<0.01	<0.01	<0.01	<0.01	<0.01	>0.10	<0.01	>0.10
Eumetabolic subjects given both diethylstilbestrol and estradiol benzoate (n = 5)											
Mean	Control		9.2	6.8	10.2	0.95	5.5	505	52.2	54.4	+5
Mean	Diethylstilbestrol	33	9.5	8.9	7.9	0.75	6.7	641	51.8	80.7	+7
Mean	Estradiol benzoate	33	9.0	8.5	8.2	0.75	6.9	617	51.5	75.1	+4
SEMD‡ and p values	Control vs diethylstilbestrol		0.4 >0.10	0.3 <0.01	0.3 <0.01	44 <0.02	0.3 <0.01	40 0.05	2.9 >0.10	2.9 <0.01	3 >0.10
	Control vs estradiol benzoate		0.2 >0.10	0.4 <0.02	0.6 <0.05	60 <0.05	0.3 <0.01	37 <0.10	5.2 >0.10	4.4 <0.01	3 >0.10
SEMD‡ and p values	Diethylstilbestrol vs estradiol benzoate		0.4 >0.10	0.4 >0.10	0.4 <0.10	69 >0.10	0.3 >0.10	54 >0.10	6.2 >0.10	3.7 >0.10	3 >0.10

* For explanation of abbreviations, see Appendix.
† Per cent radioactivity bound to TBG at a concentration of added thyroxine of 0.475 μg/ml.
‡ Standard error of the mean difference.

Eumetabolic subjects. Previously described effects of large doses of estrogen on PBI (13, 18) and on the binding activity of TBG (13) were confirmed in the present study. Increases in PBI during estrogenic therapy occurred not only in patients with intact thyroidal function, but also in patients with treated myxedema. For the entire group, mean values of the PBI increased from 5.8 to 7.6 μg per 100 ml. For the patients with treated myxedema alone, PBI increased significantly ($p < 0.001$) from a mean of 5.5 to 7.1 μg per 100 ml. Concomitantly, in sera enriched with stable thyroxine at a concentration of 47.5 μg per 100 ml, the proportion of radioactive thyroxine bound to TBG increased from a mean of 54.4 to 79.5 per cent.

In ten paired observations, diethylstilbestrol increased the mean thyroxine distribution space (TDS) from 8.8 to 9.3 L, a change of borderline statistical significance. Mean thyroxine half-time ($T_{\frac{1}{2}}$) also increased from 6.6 to 8.9 days, while the fractional rate of turnover of hormone (k) correspondingly decreased from 10.7 to 7.9 per cent per day. Despite the increase in TDS, mean daily thyroxine clearance (C) decreased from 0.94 to 0.73 L per day. The product of daily hormonal clearance rate (C) and hormonal concentration (PBI) represents the quantity of hormonal iodine removed from the peripheral thyroxine space daily (D). As a result of reciprocal changes in its constituent functions, D remained unchanged during estrogenic therapy in the group as a whole. Small, random differences between control and treatment values of D, noted in individual patients, were probably the result of errors intrinsic to the technique of measurement. Following equilibration of PBI and TBG, basal oxygen consumption during estrogenic treatment did not differ from control values.

As a result of increases in both TDS and PBI, the mean quantity of exchangeable (extrathyroidal) hormonal iodine (ETT) increased significantly from 506 to 699 μg during administration of estrogen. Derived values for the fraction of total hormonal disposal accounted for by fecal excretion (F_{max}) did not change significantly for the group as a whole.

In the five patients who received both types of estrogen, significant differences in the effects of

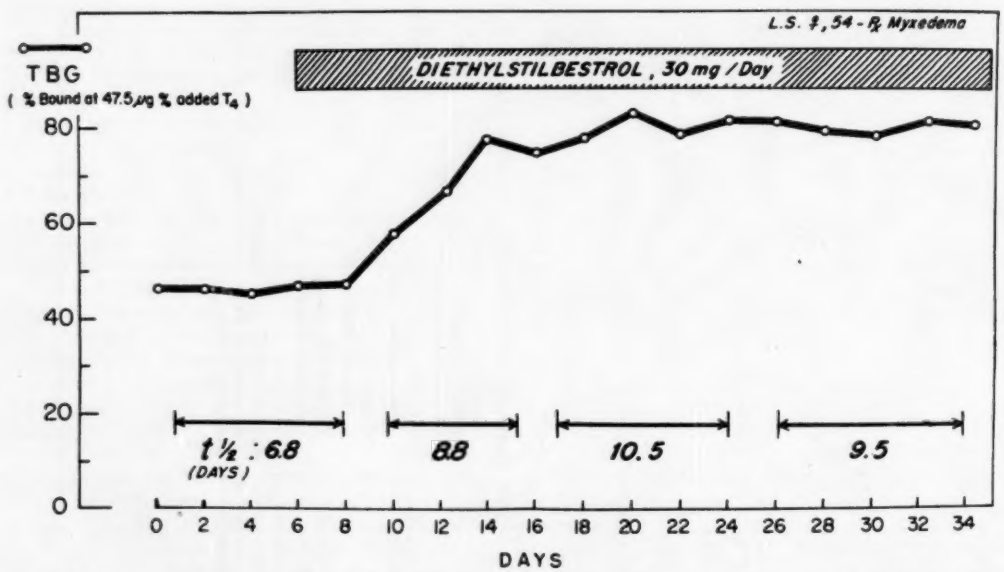

FIG. 1. THE RELATIONSHIP OF ESTROGEN-INDUCED INCREASES IN THE BINDING AVIDITY OF TBG TO CHANGES IN PERIPHERAL TURNOVER OF THYROXINE IN A PATIENT WITH TREATED MYXEDEMA (PATIENT 6). ●—● Indicates the percentage of added I^{131}-labeled thyroxine migrating with TBG during paper electrophoresis of serum enriched with stable thyroxine at a concentartion of 47.5 μg per 100 ml.

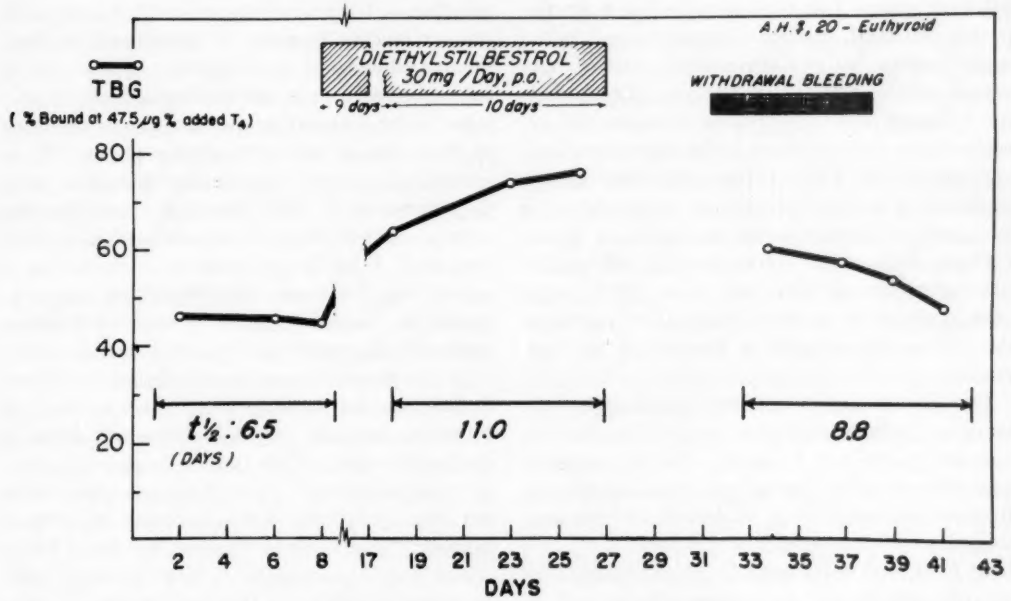

FIG. 2. THE RELATIONSHIP OF ESTROGEN-INDUCED INCREASES IN THE BINDING AVIDITY OF TBG TO CHANGES IN PERIPHERAL TURNOVER OF THYROXINE IN A EUTHYROID PATIENT (PATIENT 9).

diethylstilbestrol and estradiol benzoate could not be discerned.

Studies performed in Patient 6 throughout the 24 day period following initiation of estrogenic therapy, and in Patient 9 following cessation of treatment, are depicted in Figures 1 and 2. In Patient 6 the control rate of peripheral turnover of thyroxine was 10.2 per cent per day and was followed in successive weeks by rates estimated to be 7.9, 6.6, and 7.3 per cent per day. Increases in thyroxine-binding by TBG were detected on the fourth day of administration of diethylstilbestrol, and reached a maximum after 14 days. In Patient 9, following a pretreatment rate of 10.7 per cent per day, the fractional rate of turnover of thyroxine decreased to 6.6 per cent per day during continued estrogenic therapy. After cessation of treatment, increased thyroxine-binding by TBG persisted for a time and turnover rate remained retarded at 7.3 per cent per day, although withdrawal vaginal bleeding had occurred.

Administration of diethylstilbestrol to the women with Graves' disease was also accompanied by slowing of the fractional rate of peripheral turnover of thyroxine (Table I). In all four patients, this rate was as slow or slower than that subsequently observed after antithyroid treatment. In Patients 12 and 13, estrogenic therapy was accompanied by amelioration of symptoms of thyrotoxicosis and by reduction of basal metabolic rates to normal. In these patients, in contradistinction to the two patients who experienced no symptomatic benefit, PBI was unchanged or decreased during estrogenic therapy as compared to control periods. Correspondingly, calculated values for daily hormonal disposal (D) in these two women were markedly reduced, approaching values obtained after treatment with antithyroid drugs.

DISCUSSION

In agreement with the recently published findings of Engbring and Engstrom (19), the present studies seem clearly to indicate that the prolonged administration of either natural or synthetic estrogenic hormones is associated with pronounced changes in the peripheral metabolism of thyroxine. These include a decrease in the fractional rate of turnover of thyroxine and hence in the rate of clearance of the hormone from its peripheral dis-

tribution space. Concomitantly, both the concentration and total quantity of hormone within the space increase, so that the absolute rate of hormonal disposal remains unchanged. These studies, however, were undertaken to assess the kinetic effects of an increase in the thyroxine-binding activity of TBG. Estrogens were merely employed as a means of altering TBG. Thus, it is critical to distinguish between changes in the kinetics of thyroxine metabolism which resulted from alterations in TBG and those which might have arisen from a direct effect of estrogens on the cellular degradation or disposal of the hormone.

Certain data suggest that estrogens may directly influence cellular oxidative metabolism and its response to thyroid hormone. While estrogens generally stimulate genital structures, several estrogens have been found to depress oxygen consumption of homogenates of rat brain (20) and liver (21), and of fresh rat liver mitochondria incubated with Krebs' cycle intermediates and diphosphopyridine nucleotide (22). Animals treated with estrogen have been reported to be unusually resistant to large doses of thyroid hormone (23–26), and diethylstilbestrol has been shown to hasten the return of oxygen consumption to normal following cessation of thyroid feeding in rats (27). However, it is not known whether such effects on oxidative metabolism might result from or lead to changes in the intracellular metabolism of thyroxine. More direct efforts to evaluate this question have led to contradictory conclusions, since estrogens have had variable effects on the degradation of thyroxine by cellular preparations, *in vitro* (28, 29). Finally, *in vivo,* estrone does not affect the disappearance of thyroxine from the plasma of rats or rabbits (30, 31).

Thus, it is possible that the slowing of thyroxine turnover induced by estrogens during the present studies may have resulted either from a direct effect of these hormones on the cellular degradation of thyroxine or from an effect on cellular oxidative metabolism reflected secondarily in delayed thyroxine turnover. However, there is no substantial evidence to suggest that this was the case. On the other hand, there is considerable evidence to indicate that the slowing of thyroxine turnover herein produced resulted from an increase in the binding activity of TBG. *In vitro,* the static distribution of thyroxine between cellular and extracellular binding proteins is conditioned by their relative abundance and binding avidity (5, 6, 32). An increase in the binding activity of proteins in the suspending medium favors retention of thyroxine in the extracellular phase. If, as seems likely, similar relationships pertain *in vivo,* then increases in TBG should decrease the proportion of peripheral hormone within cellular confines. Since the degradation of thyroxine is an intracellular event, this should, in turn, diminish the fractional rate of turnover of hormone within the thyroxine space as a whole (12, 33).

In the present experiments, studies in Patient 6 illustrate the rapidity with which slowing of thyroxine turnover may follow the exhibition of diethylstilbestrol. This rapid change might suggest a direct cellular effect of the estrogen were it not that circulating TBG increased with equal rapidity. In Patient 9, retarded turnover of thyroxine and augmentation of TBG persisted after cessation of estrogenic therapy. Although withdrawal vaginal bleeding had occurred in this interval, it remains possible that diminishing but physiologically significant stores of estrogen persisted during that time.

Additional evidence that estrogenic effects on the fractional turnover of thyroxine result from altered hormonal binding has recently been obtained in two patients whose sera demonstrated spontaneous abnormalities in the thyroxine-binding capacity of TBG. The first patient, recently reported by Beierwaltes and Robbins (34), displayed an apparently idiopathic increase in TBG, associated with abnormalities in the turnover of thyroxine remarkably similar to those induced by estrogens in the present studies. The plasma of the second patient was virtually devoid of TBG. Administration of estrogen induced edema and gynecomastia, but the binding capacity of TBG was not increased and the kinetics of thyroxine metabolism were unaltered (14).

From the foregoing observations in isolated cellular systems and in patients, it would appear that the effects of estrogenic hormones on the kinetics of thyroxine metabolism are indeed the result of concomitantly induced changes in the binding activity of TBG. Rate limitations imposed by TBG on the removal of thyroxine from its peripheral distribution compartment might be exerted

through effects on either the excretory or the degradative disposal of hormone, or both. Since urinary losses of intact thyroxine contribute but very slightly to the total disposal of the hormone (35, 36), decreased urinary excretion of iodine could not have accounted for the slowing of fractional turnover rates induced by estrogens. In contrast, fecal excretion of iodinated organic compounds contributes significantly to total hormone turnover (7, 37). Accordingly, estimates of the fraction of hormone removed from the thyroxine space by fecal excretion before and during estrogenic therapy were made in seven patients. Variable, seemingly random alterations in calculated values for the fractional removal of hormone by the fecal route (F_{max}) occurred. Such variations may have been attributable to inaccuracies in the estimation of F_{max}. Nevertheless, fractional fecal excretion of thyroxine appeared to decrease during estrogenic therapy in five out of seven patients. For the group as a whole, however, changes in F_{max} were not statistically significant. Values for the rate of fecal clearance of hormone, calculated as the product of the hormonal clearance rate and the fecal fraction, were decreased in six of seven patients, and for the group as a whole the decrease was significant at the 5 per cent level. Nonexcretory, presumably degradative clearances, calculated as the difference between total and fecal clearance rates were diminished during estrogenic therapy in all patients. For the group as a whole, this change was highly significant ($p < 0.01$). It would thus appear that both the degradative metabolism and the fecal excretion of thyroxine are limited by the association between thyroxine and TBG. This conclusion is consonant with the observations of Rasmussen (38) and of Myant (39), which indicate that the fecal or biliary elimination of thyroxine is increased when hormonal binding sites in the plasma are more nearly saturated than normal.

The present studies can be divided into three periods: *1*) a control period in which PBI and TBG were constant; *2*) a period of two to four weeks beginning shortly after the administration of estrogen, in which PBI and TBG were rising;[4] and *3*) a period in which PBI and TBG, though increased, were again constant, despite continued administration of estrogen. In patients with treated myxedema, hormonal supplies were constant throughout the period of observation. Constancy of PBI's during the control period would indicate that influx and removal of hormone were equal. Since this was also true during the final period, hormonal disposal must then have returned to control levels. This was confirmed, within the limits of experimental error, by kinetic studies with labeled thyroxine. These further indicated that the quantity of hormone removed by both fecal and degradative routes had returned to normal by that time.

The second period may be considered a phase during which the peripheral metabolism of hormone was re-adjusting to the effects of estrogenic therapy. During this time, hormonal removal must have declined below control values, since both the concentration and total content of hormone within the thyroxine space were increasing. Presumably, this reflected a sparing of hormone by both fecal and degradative mechanisms. If so, then the quantity of hormone available to intracellular degradative sites must have declined below control values. The magnitude of this tissue deficit of hormone at any moment in time cannot be assessed. However, for the entire period of approximately one month's duration, a mean positive balance of 193 μg of hormonal iodine per patient was obtained. If fractional removal of hormone declined abruptly after initiation of estrogenic therapy while the increase in PBI occurred more slowly, then the daily deficit of hormone within the tissues would have been relatively large during early portions of the period and would have become progressively smaller as re-equilibration was approached. If, however, fractional turnover of hormone declined slowly and PBI increased *pari passu*, then there would have

[4] In an earlier study from this laboratory (13), a consistent increase in PBI was not found during estrogenic therapy in patients with treated myxedema. However, the present studies should afford a more reliable index of estrogenic effects in that a larger number of patients was studied and PBI's were determined more frequently. In addition, it can be demonstrated mathematically that when hormonal supplies are constant and fractional turnover of hormone declines, the magnitude of the resulting increase in PBI is proportional to the control PBI value (40). Accordingly, patients in the present study were maintained on somewhat larger doses of thyroid hormone in order to insure the establishment of control PBI's well within the normal range.

occurred only a slight daily deficit in hormonal delivery of relatively uniform magnitude throughout the period. Since TBG increased progressively throughout this phase, the latter possibility seems the more likely.

While, in patients with treated myxedema, the postulated sequence of events is relatively simple, in patients with functioning thyroid glands it is potentially more complex. Here, it might be anticipated that the reduction in hormonal flux to the tissues would result in enhanced secretion of thyrotropin, since hypophyseal homeostatic mechanisms seem directed toward maintaining constant supplies of thyroid hormone to peripheral tissues (41). Consequent stimulation of the synthesis and release of thyroid hormone would serve to diminish the deficit in peripheral hormonal supplies and to hasten return to a steady state. However, it is questionable whether such an increase in thyroidal function actually occurs. Diethylstilbestrol has been found to increase the rate of release of thyroidal I^{131} of euthyroid patients undergoing hypophyseal suppression by triiodothyronine (42). On the other hand, evidence of hypophyseal activation has not been obtained, since repeated efforts to demonstrate an increase in the thyroidal uptake of iodine during therapy with diethylstilbestrol have failed (18, 43). It may be that the deficit in peripheral hormonal supplies induced by the administration of estrogens is never sufficiently great to activate secretion of TSH. Alternatively, previous studies may have been performed during the final phase of the response to estrogens, when a new steady state had been achieved.

Findings in patients with thyrotoxicosis were of interest in several respects. Previously reported abnormalities in the kinetics of thyroxine metabolism were confirmed. Thus, three of four patients displayed accelerated fractional rates of thyroxine turnover both prior to treatment (7, 37, 44) and following restoration of a eumetabolic state by propylthiouracil (45). During administration of diethylstilbestrol, increased binding activity of TBG occurred in all patients. Concomitantly, fractional rates of turnover became at least as slow as those found subsequently during antithyroid therapy. In Patients 14 and 15, the remaining findings were similar to those seen in eumetabolic subjects, in that PBI's increased, hormonal clearance rates declined, daily hormonal disposal remained essentially unaltered, and metabolic status was unchanged.

In Patients 12 and 13, strikingly different findings obtained. Here, despite marked decreases in hormonal clearance rates, PBI's failed to increase. This could have occurred only if the flux of hormone from the thyroid gland to the periphery had declined during administration of estrogen. From the data of other workers (46), it would appear that estrogens may slow the rate of release of glandular iodine in some patients with thyrotoxicosis. Because of the decreased hormonal clearance rates and the unchanged PBI's, daily hormonal disposal in these patients decreased to values approaching those found during antithyroid therapy. At the same time, there occurred a marked amelioration of the signs and symptoms of thyrotoxicosis and reduction in basal metabolic rate. This favorable response was reminiscent of the occasional amelioration of thyrotoxicosis by estrogens reported by earlier workers (47). From the present data, it is evident that the symptomatic remission of thyrotoxicity, which estrogens may occasionally induce, results from thyroidal and peripheral effects reflected in decreased hormonal disposal, rather than from direct anti-oxidative effects within the tissues. However, those factors which account for the variability in the thyroidal response to estrogens, as seen in this and earlier studies, remain an intriguing enigma.

SUMMARY

The effects of diethylstilbestrol and of estradiol benzoate upon the kinetics of thyroxine metabolism have been assessed in patients with intact thyroidal function, treated myxedema, or Graves' disease.

In all subjects, estrogen induced an increase in the binding avidity of the thyroxine-binding globulin (TBG), which was accompanied by marked retardation of the fractional rate of peripheral turnover of I^{131}-labeled thyroxine. In eumetabolic subjects, concentration of protein-bound iodine (PBI) increased sufficiently to restore the daily disposal of hormonal iodine to normal values.

Two of four thyrotoxic patients failed to demonstrate expected increases in the concentration of PBI, and exhibited amelioration of symptoms and of hypermetabolism during administration of es-

trogen. During this period, daily hormonal disposal rates approached values found subsequently during restoration of eumetabolism by propylthiouracil.

These observations are consistent with the hypothesis that thyroxine-binding by TBG exerts a rate-limiting effect upon the peripheral metabolism of this hormone.

APPENDIX

In the foregoing manuscript, the following terms have been employed. These were described in detail in an earlier communication (7).

TDS, thyroxine distribution space; the virtual volume of body fluids through which exchangeable thyroxine would be distributed were it present throughout at the same concentration at which it exists in the plasma.

PBI, concentration of hormonal iodine in the plasma, and, by definition, the geometrical mean concentration of hormonal iodine in the TDS; assumed to represent solely iodine in thyroxine.

ETT, extrathyroidal thyroxine; the total quantity of thyroxine within the TDS, in terms of its content of iodine.

k, Fractional rate of turnover of thyroxine; the proportion of ETT removed from the TDS per unit time.

$T_{\frac{1}{2}}$, thyroxine half-time; the time required for half of the ETT to be removed from the TDS.

C, thyroxine clearance rate; the volume of plasma which contains an amount of thyroxine equal to that removed from the TDS per unit time. Defined as "volume turnover, V" in an earlier communication (7).

D, hormonal disposal rate; the quantity of hormone removed from the TDS per unit time, in terms of its content of iodine.

F_{max}, the proportion of D accounted for by fecal excretion of hormone; fecal clearance, the volume of plasma which contains a quantity of hormone equal to that lost in the feces per unit time; degradative clearance, the volume of plasma which contains a quantity of hormone equal to that removed by nonexcretory pathways per unit time; fecal excretion, the quantity of hormone excreted in the feces per unit time; degradative rate, the quantity of hormone removed by nonexcretory pathways per unit time.

$ETT = PBI \times TDS$. $C = TDS \times k$. $D = C \times PBI = ETT \times k$. $T_{\frac{1}{2}} = 0.693/k$. Fecal clearance $= C \times F_{max}$. Degradative clearance $= C -$ fecal clearance. Fecal excretion = fecal clearance $\times$ PBI $= D \times F_{max}$. Degradative rate = degradative clearance $\times$ PBI $= D -$ fecal excretion.

REFERENCES

1. Gordon, A. H., Gross, J., O'Connor, D., and Pitt-Rivers, R. Nature of the circulating thyroid hormone-plasma protein complex. Nature (Lond.) 1952, **169**, 19.
2. Larson, F., Deiss, W. P., and Albright, E. C. Localization of protein-bound radioactive iodine by filter paper electrophoresis. Science 1952, **115**, 626.
3. Robbins, J., and Rall, J. E. The interaction of thyroid hormones and protein in biological fluids. Recent Progr. Hormone Res. 1957, **13**, 161.
4. Ingbar, S. H. Pre-albumin: A thyroxine-binding protein of human plasma. Endocrinology 1958, **63**, 256.
5. Crispell, K. R., and Coleman, J. A study of the relative binding capacity of plasma proteins, intact human red cells, and human red cell stroma for radioactive I-131 labeled L-thyroxine. J. clin. Invest. 1956, **35**, 475.
6. Freinkel, N., Ingbar, S. H., and Dowling, J. T. The influence of extracellular thyroxine-binding protein upon the accumulation of thyroxine by tissue slices. J. clin. Invest. 1957, **36**, 25.
7. Ingbar, S. H., and Freinkel, N. Simultaneous estimation of rates of thyroxine degradation and thyroid hormone synthesis. J. clin. Invest. 1955, **34**, 808.
8. Volwiler, W., Goldsworthy, P. D., MacMartin, M. P., Wood, P. A., Mackay, I. A., and Fremont-Smith, K. Biosynthetic determination with radioactive sulfur of turn-over rates of various plasma proteins in normal and cirrhotic man. J. clin. Invest. 1955, **34**, 1126.
9. Robbins, J., Rall, J. E., and Petermann, M. L. Thyroxine-binding by serum and urine proteins in nephrosis. Qualitative aspects. J. clin. Invest. 1957, **36**, 1333.
10. Federman, D. D., Robbins, J., and Rall, J. E. Effects of methyl testosterone on thyroid function, thyroxine metabolism, and thyroxine-binding protein. J. clin. Invest. 1958, **37**, 1024.
11. Richards, J. B., Dowling, J. T., and Ingbar, S. H. Alterations in the plasma transport of thyroxine in sick patients and their relation to the abnormality in Graves' disease (abstract). J. clin. Invest. 1959, **38**, 1035.
12. Ingbar, S. H., and Freinkel, N. The thyroid hormone *in* Hormones in the Blood, H. N. Antoniades, Ed. Boston, Little, Brown & Co. In press.
13. Dowling, J. T., Freinkel, N., and Ingbar, S. H. Effect of diethylstilbestrol on the binding of thyroxine in serum. J. clin. Endocr. 1956, **16**, 1491.
14. Ingbar, S. H. Unpublished observations.
15. Dowling, J. T., Freinkel, N., and Ingbar, S. H. Thyroxine-binding by sera of pregnant women, newborn infants, and women with spontaneous abortion. J. clin. Invest. 1956, **35**, 1263.
16. Robbins, J., and Nelson, J. H. Thyroxine-binding by serum protein in pregnancy and in the newborn. J. clin. Invest. 1958, **37**, 153.
17. Snedecor, G. W. Statistical Methods Applied to Experiments in Agriculture and Biology, 4th ed. Ames, Iowa, The Iowa State College Press, 1946.

18. Engstrom, W. W., and Markardt, B. Influence of estrogen on thyroid function. J. clin. Endocr. 1954, **14**, 215.
19. Engbring, N. H., and Engstrom, W. W. Effects of estrogen and testosterone on circulating thyroid hormone. J. clin. Endocr. 1959, **19**, 783.
20. Gordan, G. S., and Elliott, H. W. The action of diethylstilbestrol and some steroids on the respiration of rat brain homogenates. Endocrinology 1947, **41**, 517.
21. Guidry, M. A., Segaloff, A., and Altschul, A. M. The effect of a-estradiol, some of its esters and estrone on the respiration of tissue homogenates. Endocrinology 1952, **50**, 29.
22. Wade, R., and Jones, H. W., Jr. Effect of progesterone on oxidative phosphorylation. J. biol. Chem. 1956, **220**, 553.
23. Bodansky, M., and Duff, V. B. The influence of pregnancy on resistance to thyroxine, with data on the creatine content of the maternal and fetal myocardium. Endocrinology 1936, **20**, 537.
24. Danforth, D. N., Greene, R. R., and Ivy, A. C. The effect of female sex hormones upon the oxygen consumption rate of normal rats, and upon the tolerance to desiccated thyroid. Endocrinology 1937, **21**, 361.
25. Sherwood, T. C. The relation of estrogenic substances to thyroid function and respiratory metabolism. Amer. J. Physiol. 1938, **124**, 114.
26. Doisy, R. J., and Lardy, H. A. Effect of certain steroids on metabolic rate of hyperthyroid rats. Amer. J. Physiol. 1957, **190**, 142.
27. Sherwood, T. C. The effect of stilbestrol on the basal metabolism of experimental hyperthyroid rats. Endocrinology 1940, **26**, 693.
28. Cruchaud, S., Vannotti, A., Mahaim, C., and Deckelmann, J. The in-vitro effect of methylthiouracil and oestradiol monophosphate on the conversion of thyroxine to triiodothyronine by kidney slices. Lancet 1955, **2**, 906.
29. Yamazaki, E., and Slingerland, D. W. The *in vitro* metabolism of thyroxine, triiodothyronine and their acetic and propionic acid analogues. Endocrinology 1959, **64**, 126.
30. Feldman, J. D. Effect of estrogen on the peripheral utilization of thyroid hormones. Amer. J. Physiol. 1957, **188**, 30.
31. Beckers, C., Lulling, J., and de Visscher, M. Influence de la folliculine sur le métabolisme de la radiothyroxine. Acta endocr. (Kbh.) 1959, **30**, 242.
32. Hamolsky, M. W., Stein, M., and Freedberg, A. S. The thyroid hormone-plasma protein complex in man. II. A new *in vitro* method for study of "uptake" of labelled hormonal components by human erythrocytes. J. clin. Endocr. 1957, **17**, 33.
33. Ingbar, S. H., and Freinkel, N. Regulation of the peripheral metabolism of the thyroid hormones. Recent Progr. Hormone Res. In press.
34. Beierwaltes, W. H., and Robbins, J. Familial increase in the thyroxine-binding sites in serum alpha globulin. J. clin. Invest. 1959, **38**, 1683.
35. Myant, N. B., and Pochin, E. E. The metabolism of radiothyroxine in man. Clin. Sci. 1950, **9**, 421.
36. Rall, J. E. Iodine compounds in the blood and urine of man. J. clin. Endocr. 1950, **10**, 996.
37. Berson, S. A., and Yalow, R. S. Quantitative aspects of iodine metabolism. The exchangeable organic iodine pool, and the rates of thyroidal secretion, peripheral degradation and fecal excretion of endogenously synthesized organically bound iodine. J. clin. Invest. 1954, **33**, 1533.
38. Rasmussen, H. Thyroxine metabolism in the nephrotic syndrome. J. clin. Invest. 1956, **35**, 792.
39. Myant, N. B. Relation between the biliary clearance rate of thyroxine and the binding of thyroxine by the serum proteins. J. Physiol. (Lond.) 1957, **135**, 426.
40. Ingbar, S. H., and Freinkel, N. ACTH, cortisone and the metabolism of iodine. Metabolism 1956, **5**, 652.
41. Ingbar, S. H. Implications of thyroxine turnover in man *in* Progress in Clinical Endocrinology, E. B. Astwood, Ed. New York, Grune & Stratton, Inc. In press.
42. Dowling, J. T., Ingbar, S. H., and Freinkel, N. The role of thyroxine-binding interactions in the thyroidal release of hormone. Clin. Res. 1958, **6**, 24.
43. Dowling, J. T., Ingbar, S. H., and Freinkel, N. Failure of diethylstilbestrol to affect thyroidal accumulation and renal clearance of I^{131}. J. clin. Endocr. 1959, **19**, 1245.
44. Sterling, K., and Chodos, R. B. Radiothyroxine turnover studies in myxedema, thyrotoxicosis, and hypermetabolism without endocrine disease. J. clin. Invest. 1956, **35**, 806.
45. Ingbar, S. H., and Freinkel, N. Studies of thyroid function and the peripheral metabolism of I^{131}-labeled thyroxine in patients with treated Graves' disease. J. clin. Invest. 1958, **37**, 1603.
46. Solomon, D. H. Factors affecting the fractional rate of release of radioiodine from the thyroid gland in man. Metabolism 1956, **5**, 667.
47. Starr, P., and Patton, H. Observations of remissions in hyperthyroidism induced by pregnancy urine extract. Ann. intern. Med. 1936, **8**, 825.

BILIARY TRANSPORT AND HEPATIC STORAGE OF SULFOBROMOPHTHALEIN SODIUM IN THE UNANESTHETIZED DOG, IN NORMAL MAN, AND IN PATIENTS WITH HEPATIC DISEASE *

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Since its introduction in 1924 by Rosenthal and White (1), the dye sulfobromophthalein sodium (BSP) has proven useful not only for clinical purposes but also for physiological investigations of splanchnic vascular perfusion, hepatocellular function, and biliary secretory mechanisms. It has been shown that removal of BSP from the plasma by the liver depends upon two distinct processes: *1)* accumulation of dye in hepatic cells and *2)* active secretion into the biliary tract.

Hepatocellular accumulation or "storage" of BSP was first suggested by the demonstration of a marked delay between removal from the blood and excretion into the bile after the dye was administered intravenously to dogs (2, 3), and a similar mechanism in man was inferred from a study of the hepatic removal of successive doses of BSP (4). The accumulation of a related phthalein dye, fluorescein, in hepatic polygonal cells has actually been visualized in living frog and rat livers by the technique of fluorescence microscopy (5, 6). In the case of another phthalein dye, I^{131}-labeled rose bengal, an accumulation or "storage phase" is apparent in the curves obtained by external counting over the liver after intravenous injection (7), and high concentration within liver cells has been confirmed by radioautography (8). Although it has been suggested that accumulation of BSP may proceed until there is complete "saturation" of intrahepatic storage sites (4, 9), more recent evidence indicates that the process tends toward an equilibrium state in which the quantity (and hence the concentration) of BSP in the liver is directly proportional to the concentration of dye in the surrounding plasma (10). It has therefore been possible to define a BSP "relative storage capacity" (S) as the number of milligrams of BSP stored in the liver per milligram per 100 ml of plasma concentration. The value of S can be estimated by comparison of the rates of movement of BSP into the liver associated with different rates of change of plasma concentration (10). The method of calculation is described below.

The high concentrations of BSP observed in the bile (2) indicate that the ultimate excretion of this dye undoubtedly depends upon an active transport mechanism. There is convincing indirect evidence in the dog (9–11), in the rabbit (12, 13), and in man (14, 15) that the secretion of BSP into the bile is limited by a definite maximal rate, analogous to the transport maximum, or "Tm," which is demonstrable for many substances secreted by the renal tubules. For understandable technical reasons the BSP transport maximum has not, until the present, been measured directly by bile collection. Moreover, because of the nature of the BSP storage process, the rate of biliary secretion cannot be equated to the rate of hepatic removal of BSP from the plasma unless the plasma level is absolutely constant. An indirect estimation of the BSP transport maximum is possible, nevertheless, by analysis of the data obtained during determination of relative storage capacity (10).

With a method at hand for the quantitation of BSP relative storage capacity and transport maximum, it has been possible to study hepatocellular function and biliary secretion in the unanesthetized dog and to evaluate the effect of hepatic disease on these processes in man. The results are presented in this paper.

THEORETICAL CONSIDERATIONS

The hepatic removal rate of BSP at any instant must be equal to the sum of the biliary excretion rate plus the rate of accumulation (or minus the rate of deple-

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tion) of BSP in hepatic parenchymal storage. The biliary excretion rate, under conditions where the secretory transport mechanism is saturated, is equal by definition to Tm. If the number of milligrams of BSP stored in the liver cells per milligram per 100 ml of plasma BSP concentration is defined by the relative storage capacity, S, then the rate of accumulation (or depletion) of stored BSP will be equal to the product of S times the rate of change of plasma concentration ($\Delta p/\Delta t$). Thus hepatic removal rate, R, is a function of both the secretory and storage processes:

$$R = Tm + S \cdot (\Delta p/\Delta t) \quad [1]$$

where R = hepatic removal rate, milligrams per minute;
Tm = secretory transport maximum, milligrams per minute;
S = relative storage capacity, milligrams per milligram per 100 ml;
$\Delta p/\Delta t$ = rate of change of plasma concentration, milligrams per 100 ml per minute.

The value of $\Delta p/\Delta t$ can be determined by measurement of BSP concentration in serial plasma samples, and, since extrahepatic removal is small (10), "R" can be calculated by the equation $R = I - PV \cdot (\Delta p/\Delta t)$ (where I = infusion rate in milligrams per minute and PV = plasma volume in hundreds of milliliters). Thus, Equation 1 contains two unknowns, Tm and S. Solution is possible by substitution of two sets of values for R and $\Delta p/\Delta t$ obtained during two different rates of BSP infusion. In actual practice three different rates of infusion were employed in the present studies in order to test the linearity of the relationship between R and $\Delta p/\Delta t$ which is implied in Equation 1. When three determinations are available, the best solution is obtained by plotting the three values for R (ordinate) against the corresponding values of $\Delta p/\Delta t$ (abscissa). In most instances the relationship between R and $\Delta p/\Delta t$ proves to be approximately linear as predicted from Equation 1, and the best straight line through the three points is determined by the method of least squares (16). The Tm is then equal to the value of R for which $\Delta p/\Delta t = 0$, and S is equal to the slope of the least squares line.

It should be noted that the relative storage capacity, S, as defined, has the dimensions of a volume (in hundreds of milliliters). In view of the relationship between BSP storage and plasma concentration (10) the hepatic storage compartment can be considered, in a sense, as an extension of the plasma volume. When regarded in this way the present method is quite similar to one of the techniques proposed by Lewis (12) for the estimation of BSP "L_m" and "BSP fluid compartment."

METHODS

A. Procedure in dogs

Fifteen studies were conducted on 6 trained adult mongrel dogs, weighing between 16 and 30 kg, which had previously undergone splenectomy, cholecystectomy, and installation of a Thomas duodenal fistula apparatus (17). At the time of an experiment the fistula was opened to permit visualization of the Ampulla of Vater through which a no. 5 or no. 6 Fr. olive-tipped ureteral catheter was inserted and advanced about 5 or 6 cm into the common bile duct. The dog was then placed upright in a sling. Bile was collected by gravity or, when it was particularly viscous, by gentle aspiration with a tuberculin syringe. Venous blood samples were collected in heparinized tubes. After obtaining blood and bile samples for analytical blanks, BSP in 5 per cent dextrose was infused intravenously for 1 hour at a constant rate of 0.4 to 0.5 mg per minute per kg (roughly twice the anticipated BSP "Tm") using a Bowman pump. During the last 30 minutes of this infusion 4 blood samples were collected at 10 minute intervals. During the second and third hours this procedure was repeated at BSP infusion rates of approximately 0.2 to 0.3 and 0.5 to 0.6 times the initial rate, respectively. In 6 studies a rapid BSP infusion (1 to 1.5 times the initial rate) was administered during the fourth hour in order to achieve a very high plasma level. Bile was obtained continuously and collected in successive separate graduated tubes at intervals of 10 to 15 minutes. Plasma volume was measured during the course of each study using I^{131}-labeled human serum albumin. The activity of a plasma sample obtained 10 minutes after tracer injection was compared with that of an aliquot of the injectate diluted to known volume with isotope-free "carrier" plasma to avoid adsorption of tracer to glass. At the end of each study the rates of delivery of the Bowman pump were calibrated.

B. Procedure in man

Studies were conducted on 4 normal male volunteers, on 15 hospital patients without liver disease, and on 15 patients with various hepatic disorders. A second study was conducted on 2 of the normal volunteers 3.5 months after the first determination. The procedure was begun about an hour after a fat-free breakfast. After obtaining an initial blood sample BSP was infused for 1 hour at a constant rate of approximately 0.3 mg per minute per kg. Four venous blood samples were drawn at 10 minute intervals during the last 30 minutes of this infusion. This procedure was repeated at infusion rates usually 0.3 and 0.6 times the initial rate during the second and third hours, respectively. In the presence of liver disease the same procedure was followed, but all infusion rates were scaled down to 25 to 50 per cent of those employed in normal subjects. A rapid estimation of the BSP concentration in the first two blood specimens was carried out so that, if necessary, the subsequent infusion rates could be adjusted to avoid BSP concentrations outside the range of 2.5 to 15 mg per 100 ml. Plasma volume was measured during the first hour in all subjects using I^{131}-labeled human serum albumin.

C. Analytical methods

Plasma. One ml of each plasma sample was diluted by the addition of 10 ml of normal saline and 0.2 ml of

20 per cent potassium hydroxide. The optical density was determined in a Beckman DU spectrophotometer at 580 mμ. The optical density of a similarly treated plasma "blank" (obtained before the infusion of BSP) was subtracted. Standard curves for this determination were prepared by the addition of known amounts of BSP to canine plasma and to both normal and icteric human plasma. It was found that accurate results could be obtained in icteric plasma provided that 15 minutes were allowed for complete color change of bilirubin after alkalinization and provided the appropriate blank was subtracted.

Bile. The determination of BSP concentration in bile was complicated by the finding that the spectrophotometric recovery of BSP added to bile was often significantly reduced when the bile was diluted in the range of 200- to 1,000-fold. The reason for this phenomenon was not apparent, but it was found empirically that accurate measurement of BSP could always be obtained if the final dilution of bile was less than 200-fold. Since the BSP concentrations encountered in dog bile necessitated a higher dilution, this problem was circumvented by the addition of "blank" bile to the dilute bile specimens. A pooled control bile sample, obtained before BSP infusion, served as the source of the "blank" bile.

All bile samples were first diluted 100 times with distilled water. One ml of the diluted sample was then mixed with 1 ml of a fivefold dilution of the "blank" bile, 20 ml of water, and 0.4 ml of 20 per cent potassium hydroxide and the optical density determined at 580 mμ. The optical density of a similarly treated control bile was subtracted.

Infusion mixture. Concentration of BSP in the infusion mixture was determined at 580 mμ after appropriate aqueous dilution and addition of alkali.

D. Calculation of Tm and S

The values of plasma BSP concentration were plotted on a linear scale against time. The rate of change of BSP concentration ($\Delta p/\Delta t$) during the last 30 minutes of each infusion period was calculated by application of the method of least squares (16) to the 4 points obtained during that time, although in most instances a reasonably straight line through the 4 points could have been fitted by eye. A value of hepatic removal rate was determined for each infusion period, and Tm and S were calculated as described under "Theoretical Considerations."

RESULTS

A. Dogs

Tm and S. During the latter half of each period of constant BSP infusion the plasma BSP concentration (P) changed in an approximately linear fashion with respect to time (Figure 1). Thus a constant hepatic BSP removal rate was achieved during each of the three constant infusions. When the three values of R were plotted

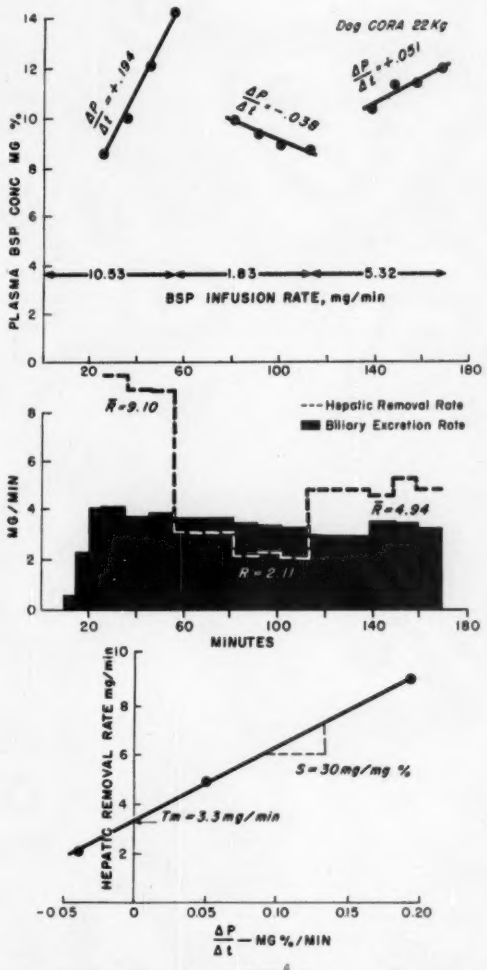

FIG. 1. ESTIMATION OF BSP[6] TM AND S IN THE DOG. Constant infusions of BSP were administered at the three rates indicated. Plasma concentration changed in linear fashion with respect to time during the latter half of each infusion period. Biliary BSP excretory rate achieved a maximal value of 4 mg per minute within 30 minutes of starting BSP and was thereafter independent of plasma concentration, although it tended to diminish gradually as the study progressed. The hepatic BSP removal rate was greater than the excretory rate during periods of increasing plasma concentration, indicating accumulation of BSP in the liver. When plasma concentration was falling, removal rate was less than excretory rate indicating net depletion of stored BSP. The relationship between $\Delta p/\Delta t$ and removal rate (R) shown below is consistent with the relationship: $R = Tm + S \cdot (\Delta p/\Delta t)$. Tm and S are calculated from the intercept ($\Delta p/\Delta t = 0$) and the slope of this line, respectively.

against the corresponding rates of change of plasma concentration ($\Delta p/\Delta t$) there was usually a linear relationship between these variables as in the example shown in Figure 1. Owing to the difficulty of maintaining a steady state, some deviation of this relationship from linearity occurred in several experiments, but in only one study (not included in the 15) was this deviation so pronounced that it would have been unreasonable to construct a straight "least squares" line through the three points.

The values of Tm and S are shown in Table I. Average values for individual dogs were computed on the basis of five studies on one dog and two studies on each of the others. In accordance with customary procedure these were arbitrarily adjusted to a body weight of 10 kg, although of course the data were insufficient to test the actual correlation with body size. The mean Tm for the six dogs was 1.9 mg per minute per 10 kg (range 1.4 to 3.2) and the mean value of S was 25 mg per mg per 100 ml per 10 kg (range 21 to 35).

Biliary BSP excretion. Control bile flow was usually between 0.05 to 0.15 ml per minute. Following the onset of BSP infusion there was invariably at least a twofold increase in flow. Thereafter spontaneous fluctuations in flow were commonly observed. The biliary excretion of BSP achieved a maximal rate within about 30 minutes of starting BSP infusion when plasma concentration exceeded 3 mg per 100 ml in all studies. Beyond this point excretory rate was independent of plasma BSP concentration and of hepatic removal rate as shown in Figure 1. Even when excessively high plasma levels (22.4 to 48.3 mg per 100 ml) were produced at the end of six studies by rapid BSP infusion there was no further increase in excretory rate, so that it was apparent that the maximal capacity for BSP transport had been achieved. The BSP excretory rate, averaged

TABLE I

BSP transport maximum (Tm), relative storage capacity (S) and biliary excretion rate in unanesthetized dogs

Dog	Date	Wt	Tm	Adjusted Tm	S	Adjusted S	BSP excretion rate*	$\frac{\text{Excretion rate}}{\text{Tm}} \times 100$
		kg	*mg/min*	*mg/min/10 kg*	*mg/mg%*	*mg/mg%/10 kg*	*mg/min*	
Norma	12/18/57	17.6	4.5	2.6	53	30	3.6	79
	1/10/58	17.6	4.4	2.5	24	13	3.4	78
	2/5/58	17.6	4.2	2.4	30	17	3.1	75
	2/26/58	17.5	3.2	1.8	44	25	3.0	95
	10/15/58	20.9	3.4	1.6	69	33	3.4	99
Average				2.2		24		
Nero	2/20/58	15.9	5.3	3.3	40	25	3.3	62
	3/7/58	15.9	4.9	3.1	48	30	3.9	81
Average				3.2		27		
Cora	3/12/58	22.0	2.8	1.2	49	22	2.5	92
	3/27/58	22.0	3.3	1.5	30	14	3.5	104
Average				1.4		18		
Tosca	5/12/58	19.5	4.0	2.1	67	35	2.8	68
	5/26/58	20.4	2.3	1.1	72	35	2.6	113
Average				1.6		35		
Isolde	6/11/58	23.6	3.9	1.6	76	32	3.6	94
	3/13/59	30.2	4.3	1.4	56	19	4.2	97
Average				1.5		25		
Dali	8/18/58	22.7	2.8	2.8	45	20	2.3	84
	11/17/58	22.7	3.9	1.7	49	22	3.6	91
Average				1.5		21		

Mean Tm 1.9 mg/min/10 kg (range 1.4 to 3.2)
Mean S 25 mg/mg%/10 kg (range 21 to 35)
$\frac{\text{Excretion rate}}{\text{Tm}} \times 100$: Mean ± SD 87.5 ± 13.4%

* Average BSP excretion rate from time maximum achieved to end of study.

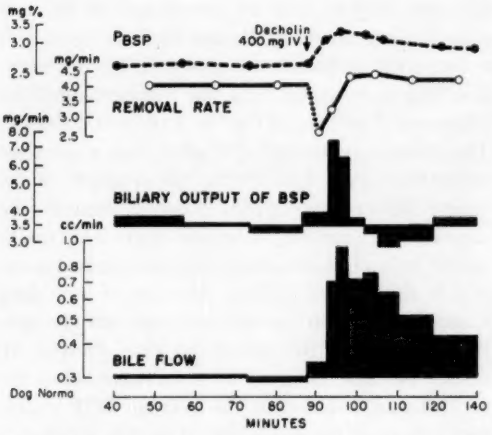

FIG. 2. EFFECT OF SODIUM DEHYDROCHOLATE (DECHOLIN). At time zero a "priming" intravenous dose of 200 mg of BSP was given and a constant infusion at 4.04 mg per minute was started. Administration of 400 mg of Decholin at the time indicated resulted in a sudden diminution in hepatic BSP removal rate, shown by the abrupt rise in plasma concentration. Bile flow increased markedly. The apparent increase in BSP excretory rate was attributable to the lag between flow and concentration changes caused by the biliary "dead space" (see text), and was followed by an apparent decrease due to the same cause. On the average, BSP excretory rate was not altered by Decholin (3.75 compared to 3.60 mg per minute), indicating continued transport of BSP from liver cells to bile during the period of reduced hepatic removal rate.

from the time the maximal rate was achieved until the end of the 3 hours of infusion, is shown in Table I. In the last column these figures are compared with the values for estimated BSP Tm. The observed excretory rate was equal, on the average, to 87.5 ± SD 13.4 per cent of the estimated Tm.

The maximal biliary excretory rate was not associated with a constant BSP concentration in bile. The latter varied considerably from study to study (range 700 to 4,210 mg per 100 ml). It also varied during the course of individual studies as bile flow changed. In one dog, for example, a spontaneous change in bile flow from 0.164 to 0.079 mg per minute was associated with a change in BSP concentration from 1,940 to 4,210 mg per 100 ml without alteration in BSP excretory rate, and similar changes were observed from time to time in all animals.

In many studies, however, there was a definite diminution in the excretory rate over the 2 to 3 hours following the establishment of the maximal rate. As in the example in Figure 1, this change was usually gradual and did not appear to be related to any specific step in the procedure. The overall change amounted to less than 15 per cent in seven studies. There was a 15 to 30 per cent diminution in five studies and a 30 to 40 per cent diminution in three. The average change for 15 studies was − 15 per cent.

Effect of sodium dehydrocholate. That the hepatic uptake and biliary excretion of BSP are independent processes was further shown by the

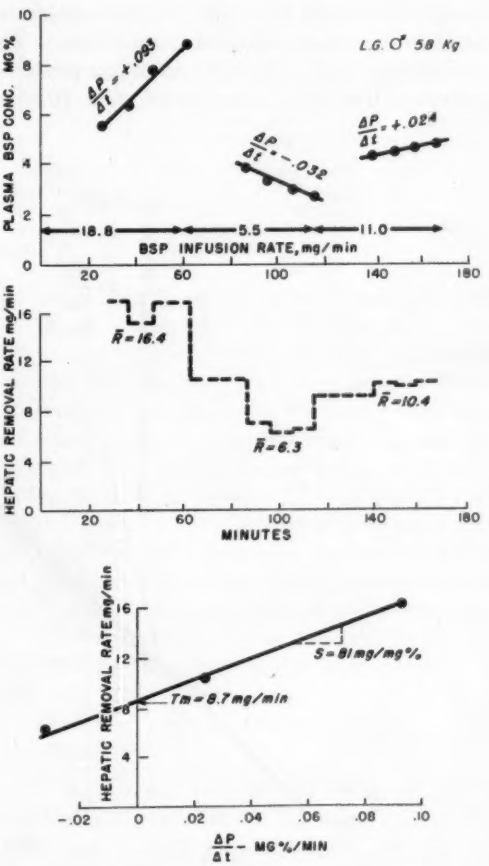

FIG. 3. ESTIMATION OF BSP TM AND S IN NORMAL MAN. Except for the actual rates of BSP infusion and the absence of data on biliary excretion, the technique and interpretation are identical with that shown in Figure 1 for the dog. (Note the very rapid removal rate during the period of increasing plasma concentration.)

effect of sodium dehydrocholate. In two dogs a "priming" intravenous dose of BSP (approximately 13 mg per kg) was followed by a constant infusion at a rate approximately equal to the previously estimated BSP Tm. By this means a relatively constant plasma concentration was achieved. After approximately 80 minutes, 400 mg of sodium dehydrocholate (Decholin) was administered intravenously. The response was identical in the two dogs and is illustrated in Figure 2. There was an abrupt diminution in hepatic removal rate (indicated by sudden increase in plasma concentration). This was followed in about 5 minutes by an increased removal rate, and finally, after about 25 minutes, by the restoration of control removal rate and plasma level. The administration of sodium dehydrocholate produced a marked transient increase in bile flow (0.30 to 0.98 and 0.36 to 0.80 ml per minute in the two dogs). Corresponding to this increase there was a reciprocal reduction in biliary BSP concentration which, however, was not observed until an additional 2 to 3 ml of the bile had been collected. This volume is somewhat smaller than a previous estimate of about 5 ml for the "dead space" of the canine biliary tract (18), possibly because the common duct and major hepatic ducts were maintained in a collapsed state by continuous aspiration in the present studies. Because of the "dead space" lag between flow and concentration changes, the calculated BSP excretory rate showed an abrupt increase followed by a decrease below the control rate. However, the average BSP excretory rate over the entire period of dehydrocholate effect did not differ significantly from the control excretory rate despite the inhibition of removal of

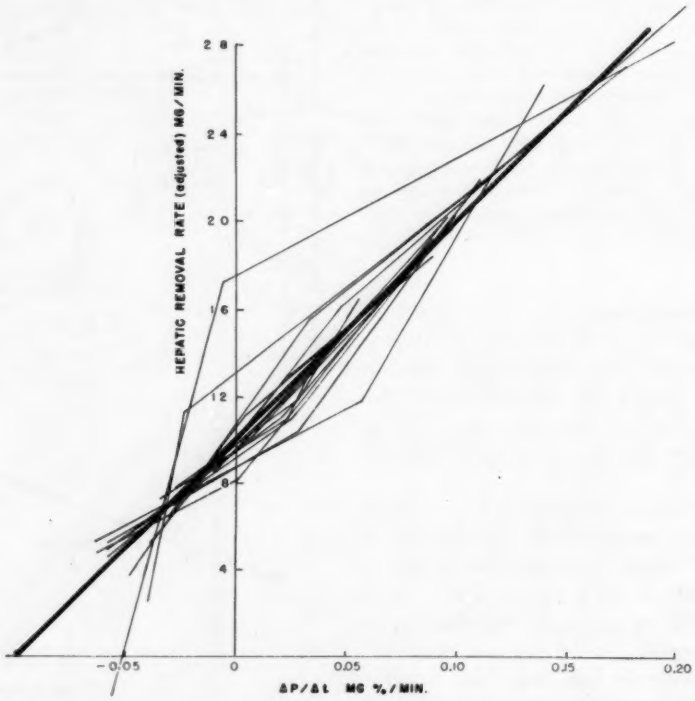

FIG. 4. RELATIONSHIP BETWEEN ADJUSTED BSP REMOVAL RATE (R′) AND $\Delta p/\Delta t$ IN 19 NORMAL SUBJECTS. Actual values of removal rate have been adjusted (see text) so that the three points ($\Delta p/\Delta t$, R′) for each subject fall about the line $R' = 10 + 100 \cdot (\Delta p/\Delta t)$. In most subjects the relationship between removal rate and rate of change of plasma concentration is close to the postulated straight line.

TABLE II

BSP transport maximum (Tm) and relative storage capacity (S) in normal human subjects

Subject	Sex	Age	Wt	Surface area*	Tm	S	Diagnosis
			kg	*m²*	*mg/min*	*mg/mg%*	
S.B.	M	44	69	1.88	7.9	115	Normal
H.W.	M	33	69	1.91	12.6	50	Normal
					11.4	77	Repeat after 3.5 months
S.V.	M	29	65	1.83	11.0	58	Normal
					11.9	51	Repeat after 3.5 months
W.C.	M	29	66	1.86	9.9	106	Normal
J.D.	M	44	67	1.71	8.0	27	Duodenal ulcer
J.F.	M	49	77	1.87	9.4	52	Duodenal ulcer
L.Gr.	M	28	58	1.72	8.7	81	Pneumonia, convalescent
L.Ga.	M	42	79	1.86	10.7	32	Essential hypertension
M.S.	M	61	56	1.57	6.3	50	Duodenal ulcer
J.Bo.	M	21	63	1.76	9.3	63	Spontaneous pneumothorax
A.D.	M	29	52	1.57	7.6	105	Postinfluenza
E.C.	M	19	80	1.99	12.8	53	Pneumonia, convalescent
J.M.	F	21	54	1.58	6.4	52	Asthma
C.F.	F	40	56	1.55	6.9	34	Pneumonia, convalescent
E.F.	F	32	58	1.56	5.7	78	Spontaneous pneumothorax
S.H.	F	44	86	1.92	7.5	65	Pneumonia, convalescent
J.Br.	F	44	73	1.67	8.1	23	Peptic ulcer
J.R.	F	64	51	1.54	7.5	74	Gastric ulcer
G.B.	F	22	60	1.65	7.8	70	Post phlebectomy
Tm	Males	9.5 ± SD 1.9 mg/min			S	Males	67 ± SD 28 mg/mg%
	Females	7.1 ± SD 0.8 mg/min				Females	57 ± SD 19 mg/mg%
	All subjects	8.6 ± SD 1.9 mg/min				All subjects	63 ± SD 25 mg/mg%

* Surface area estimated from height and weight by nomogram of Boothby and Sandiford (32).

BSP from plasma. In one dog BSP excretory rate following dehydrocholate injection averaged 3.75 compared with a control rate of 3.60 mg per minute, and in the other dog 4.41 compared with 4.27 mg per minute.

Enterohepatic circulation of BSP. When estimations of Tm and S are undertaken without bile collection (as in the studies in man which follow), an error might arise if a significant quantity of the excreted BSP were reabsorbed. An investigation of the magnitude of intestinal absorption of this dye was therefore carried out. In two dogs, 600 mg of BSP was administered into the duodenum (48 ml of 1,250 mg per 100 ml solution in normal saline given over a 40 minute period). Within 30 minutes after introduction of the dye measurable quantities appeared in the bile. Maximal bile concentration was achieved in 1 hour in one dog (648 mg per 100 ml) and in 2 hours in the other (535 mg per 100 ml) and decreased thereafter, reaching levels under 50 mg per 100 ml within 4 hours. There was no increase in bile flow. Of the total administered dose the fraction recovered in bile in the two dogs at the end of 2 hours was 2.6 and 2.3 per cent, and at the end of 3.5 hours 4.0 and 4.7 per cent, respectively. In another study 340 mg of BSP was dissolved in 15 ml of dog bile and administered into the duodenum. Only 1.4 per cent was recovered in the bile excreted over the next 4 hours. These observations are similar to those of Lorber and associates (19) in the anesthetized dog, and indicate a very limited interestinal absorption of BSP. Reabsorption of the BSP conjugates normally excreted in the bile may be even more limited. When bile (containing 350 mg of BSP) was collected from a dog receiving an intravenous BSP infusion and administered into the duodenum of another dog, no BSP was detectable in the bile excreted by the latter animal over the next 3 hours.

B. Normal man

As in the dog, linear rates of change of plasma BSP concentration were observed during the latter half of each infusion period (Figure 3). The values of Tm and S were calculated as described under "Theoretical Considerations" and as illustrated in Figure 3. The relationship between R and $\Delta p/\Delta t$ proved to be approximately linear in most instances. In order to illustrate

this relationship on a single composite graph (Figure 4), the three values of R in each subject were subjected to the operation $R' = R \cdot (100/S) + [10 - Tm \cdot (100/S)]$, so that the three points $(\Delta p/\Delta t, R')$ fell about the least squares line $R' = 10 + 100 \cdot (\Delta p/\Delta t)$. This operation does not disturb the relative positions of the three points, and it can be seen from Figure 4 that the actual relationship between R and $\Delta p/\Delta t$ adhered rather closely to the postulated straight line in most subjects. Deviations from linearity appeared to be entirely random and can probably be attributed to methodological errors or failure to maintain a steady state for 3 hours.

In four normal males the average Tm was 10.3 mg per minute and S was 85 mg per mg per 100 ml. In eight hospital "normal" males (without liver disease) the average Tm was 9.1 mg per minute and S was 58 mg per mg per 100 ml. The mean for both groups was Tm = 9.5 ± SD 1.9 mg per minute and S = 67 ± SD 28 mg per mg per 100 ml. In hospital "normal" females Tm was 7.1 ± SD 0.8 mg per minute and S = 57 ± SD 19 mg per mg per 100 ml. The individual results are shown in Table II and in Figure 5. In men but not in women there was a positive correlation between Tm and body weight ($R = 0.66$, $p < 0.05$) and between Tm and surface area ($R = 0.82$, $p < 0.01$). No correlation was apparent between S and weight or between S and surface area in either sex. In view of the limited number of subjects, no definite conclusion can be drawn regarding the relationship between various indices of body size and the values of Tm and S, and therefore no empirical correction for weight or surface area was applied in man.

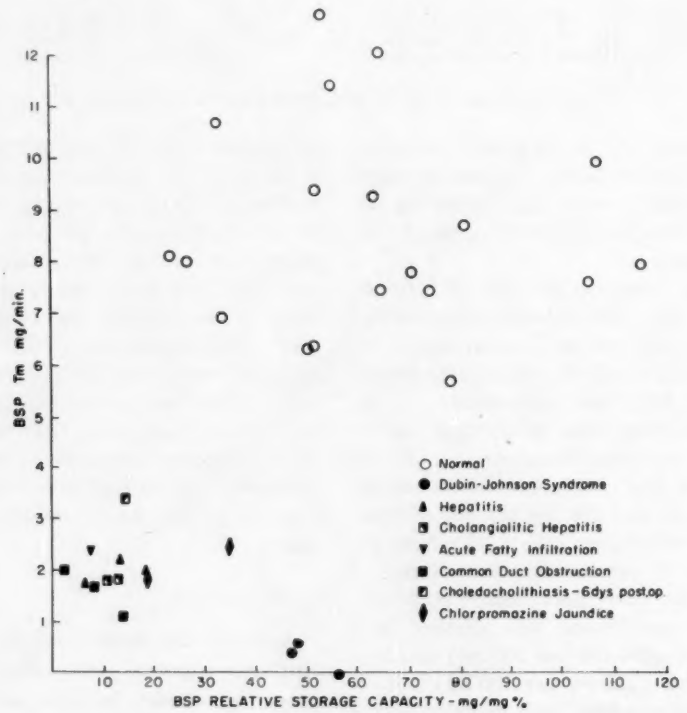

FIG. 5. TM AND S IN NORMAL HUMAN SUBJECTS AND IN PATIENTS WITH HEPATIC DISORDERS. The marked normal variation in relative storage capacity is apparent from this figure. In patients with obstructive disease as well as in those with hepatocellular disease both Tm and S were depressed. The lowest values of Tm, however, occurred in three patients with Dubin-Johnson syndrome whose capacity to store BSP was well within the normal range.

TABLE III

BSP transport maximum (Tm) and relative storage capacity (S) in patients with hepatic disorders

Subject	Sex	Age	Wt	Surface Area*	Tm	S	Diagnosis
			kg	*m²*	*mg/min*	*mg/mg%*	
J.W.	F	18	52	1.51	1.8	7	Infectious hepatitis
S.R.	M	27	59	1.75	2.0	18	Serum hepatitis
W.M.	M	65	58	1.80	2.2	14	Serum hepatitis
T.J.	F	53	82	1.80	1.8	13	Cholangiolitic hepatitis
B.M.	F	27	62	1.63	1.8	11	Cholangiolitic hepatitis
C.N.	F	28	57	1.57	2.4	8	Acute fatty infiltration
L.U.	F	46	43	1.45	1.7	8	Chronic common duct stricture
N.F.	M	51	53	1.63	2.0	2	Obstructive jaundice, carcinoma of pancreas
S.S.	F	44	58	1.62	1.1	14	Obstructive jaundice, choledocholithiasis
A.J.	M	58	81	1.88	3.4	14	6 Days after cholecystectomy and choledocholithotomy
F.S	F	48	66	1.64	2.5	35	Chlorpromazine jaundice, subsiding
M.O.	M	41	62	1.73	1.9	19	Chlorpromazine jaundice
J.L.	M	41	72	1.90	0.6	48	Dubin-Johnson syndrome
D.D.	M	28	66	1.71	(−0.9)	56	Dubin-Johnson syndrome
M.R.	F	43	43	1.36	0.3	47	Dubin-Johnson syndrome

* Surface area estimated from height and weight by nomogram of Boothby and Sandiford (32).

C. Liver disease

Patients were selected who had either primarily hepatocellular disease (hepatitis, acute fatty infiltration) or primarily obstructive disease (extrahepatic obstruction, chlorpromazine jaundice) on the theory that there might be a greater depression of S in the former and a greater depression of Tm in the latter group. However, as shown in Table III and in Figure 5 both Tm and S were reduced to approximately the same extent in patients with hepatocellular and obstructive disease. The only exception was F.S., a patient with chlorpromazine jaundice entering a period of rapid recovery at the time of the study, whose storage capacity fell within the normal range. It should be noted that all the patients with obstructive jaundice had developed symptoms at least one week before the study.

Three patients with Dubin-Johnson syndrome (chronic idiopathic jaundice) were studied, all of whom had the characteristic features of this disorder (20). In each instance the diagnosis had been established by hepatic biopsy which revealed typical centrolobular pigmentation. Patient D.D. was the nephew of M.R.[1] As shown in Table III and in Figure 5 the Tm in these patients was virtually zero whereas the storage capacity was well within the range established for normal subjects. In Patient J.L. the plasma BSP concentration was followed for 24 hours after termination of the actual study. During the first 8 hours it diminished from 19.1 to 13.6 mg per 100 ml. At the end of 16 hours the plasma level was still elevated (9.4 mg per 100 ml) indicating an extremely slow excretion of dye.

D. Toxicity

In six studies of the dog, the rapid infusion of BSP during the fourth hour increased the total administered dose to approximately 1,500 to 1,800 mg compared to the 800 to 1,000 mg given in the course of the usual 3 hour procedure. In three of these, toward the end of the fourth hour, there was a sharp diminution in bile flow and BSP excretion rate. All three appeared well when the study was terminated but began vomiting about 4 hours later. In one, whose bile was still being drained by way of the common duct catheter, the vomiting subsided within an hour and the dog was entirely well thereafter. The other two, in whom the catheter had been removed and the fistula closed, died within 6 hours of the onset of symptoms. Postmortem examination revealed severe gastrointestinal hemorrhage, most marked in the proximal small intestine. Microscopic examination of the liver in one revealed extensive

[1] We are indebted to Drs. Irwin M. Arias and Irving M. London of the Albert Einstein College of Medicine for providing us with the opportunity to study Patients D.D. and M.R.

acute central necrosis. With the possible exception of the gradual diminution in BSP excretory rate noted previously, no evidence of toxicity was ever observed in animals that received only the three BSP infusions involved in the basic procedure.

Among the patients studied, D.D. and M.R. noted transient nausea and vomiting following a meal which they ate together 8 hours after the study. Although the total amount of BSP infused in these two patients was not excessive (1,520 and 1,550 mg), the maximal plasma concentrations achieved (14.2 and 18.8 mg per 100 ml) were higher than in most other studies in man. No symptoms suggestive of toxicity were observed in any other subjects although quantities of BSP as great as 3,275 mg were infused in normal males. While the evidence for toxicity is certainly inconclusive it seems wise to adhere to the practice of "scouting" the BSP concentration in the early blood samples so that, if necessary, the subsequent infusion rates can be altered to avoid excessively high plasma levels. More prolonged or more rapid BSP infusions than those employed here should probably be avoided.

DISCUSSION

BSP secretory transport. The foregoing observations provide direct evidence in the dog that the biliary secretion of BSP involves a rate-limited process. The maximal excretory rate was achieved when the plasma BSP concentration exceeded approximately 3 mg per 100 ml, and further elevation of plasma concentration to as high as 48 mg per 100 ml did not result in any further increase in the rate of BSP excretion. Assuming a comparatively constant hepatic blood flow, this represents at least a 16-fold range in the quantity of BSP presented to the liver per unit time. In view of the variation in biliary BSP concentration encountered in these dogs, the limiting factor in BSP transport does not appear to be a fixed biliary concentration maximum as suggested by the studies of Brauer and Pessotti for anesthetized animals (3).

Although it has been shown that a major proportion of BSP appears in the bile in the form of chromotographically distinct conjugates (21–24), it is apparent that conjugation is not essential for BSP secretion since a considerable amount of unconjugated BSP is also found in all species. However, in view of the constant ratio between the amount of BSP and its conjugates, which was observed during concurrent studies (22), one cannot be certain whether the BSP Tm refers to a single transport mechanism or to the sum of separate transport mechanisms for BSP and each of its conjugates.

The magnitude of the average estimated Tm in dogs (1.9 mg per minute per 10 kg) is consistent with previous observations in this laboratory (9, 10), but is considerably lower than the L_m of 4.4 mg per minute per 10 kg estimated by Mason, Hawley and Smith (11). A possible explanation for this difference is that Mason and co-workers employed a correction for variation in plasma concentration which did not take the storage phenomenon into account. If the L_m was calculated during a period of increasing concentration (as it was in the one illustration presented by these authors), this could have led to a considerable overestimation of secretory function.

The degree of variation in the Tm from time to time in a given animal (Table I) suggests a comparable degree of variability in the biological factors which determine transport maximum. While methodological factors may have contributed to this variation, it is obvious that one must exercise caution in equating the secretory transport maximum simply to liver mass.

The finding that measured output in the bile was equal, on the average, to 87.5 per cent of the estimated Tm indicates that biliary secretion must be the major mechanism for continuous elimination of BSP despite the fact that some extrahepatic removal is known to occur (10, 25). Since the BSP conjugates in bile may have a lower molar extinction coefficient than BSP itself (21, 25), it is possible that the true biliary excretory rate of BSP was, if anything, underestimated by the spectrophotometric technique employed in the present studies. There is no satisfactory explanation for the gradual diminution in the BSP excretory rate noted in some of the studies on dogs. This may have been due to a toxic effect of the dye itself, to the depletion of some substrate essential for BSP transport, or to the depletion (by total bile collection) of substances such as bile salts which normally undergo entero-

hepatic circulation and which are involved in normal bile production (26). Any change in the actual BSP secretory rate must of course affect the calculation of Tm and S, which is based on the assumption of a 3 hour steady state with respect to BSP transport and storage. If the entire procedure could be shortened, the effect of this phenomenon, when it does occur, would be minimized. However, in the course of exploratory studies it became apparent that one cannot be sure of the establishment of a constant hepatic BSP removal rate within less than 30 minutes of the institution of a given rate of infusion. Thereafter, an additional 30 minutes is required for the reliable estimation of $\Delta p/\Delta t$ and R. Thus it appeared unwise to allow less than 1 hour for each infusion period. The magnitude of the error caused by a diminishing BSP Tm can be estimated by substituting three appropriate hypothetical sets of values for R, Tm and S into Equation 1 and obtaining the corresponding values for $\Delta p/\Delta t$. When the three sets of values for R and $\Delta p/\Delta t$ thus obtained were treated in the same manner as experimental data in estimating Tm and S, it was found that a 15 per cent diminution in BSP Tm over the course of 3 hours would lead to a 10 per cent underestimation of the initial transport maximum. The value of S would be overestimated by 6 per cent. These errors are not excessive, but data gathered over periods longer than 3 hours might well prove unreliable.

In man, the existence of a rate-limited BSP transport mechanism is indicated by the achievement of a state in which hepatic removal rate is independent of absolute plasma concentration and related only to the rate of change of plasma concentration. The mean Tm in normal subjects ($8.6 \pm$ SD 1.9 mg per minute) is considerably lower than the maximal secretory rate of 18.3 mg per minute estimated previously by Verschure (15). The latter figure was calculated by application of the formula of Mason, Hawley and Smith (11) to data obtained during a single BSP infusion at approximately 25 mg per minute. The actual data published by Verschure are, in fact, quite comparable to those observed during the rapid initial infusion employed in the method presented here. However, as shown in Figure 3, the hepatic BSP removal rate is regularly very high under the[illegible] circumstances due to the rapid accumulation of BSP in storage, so that this removal rate cannot be equated simply to the rate of secretion. In earlier work with BSP, Bradley, Ingelfinger, Bradley and Curry (14) found that an infusion rate of approximately 3 mg per minute per m^2 (or 5.2 mg per minute per 1.73 m^2) was the maximal rate at which continuously rising plasma BSP concentrations could be avoided in all their normal subjects. They suggested that this might represent an approximation to the minimal normal value of BSP Tm, and this estimate proves in fact to be roughly comparable to the lowest values of BSP Tm encountered in the present series (Table II).

Concentration of BSP in hepatic parenchyma. The accumulation of BSP within the hepatic parenchyma appears to proceed toward an equilibrium state in which the quantity (and hence the concentration) of "stored" material is directly proportional to the concentration of dye in the surrounding plasma. The liver behaves, in this respect, as though it were an extension of the plasma volume, and the relative storage capacity, S, actually has the dimensions of a "volume." However, the magnitude of S, when compared with the actual size of the liver, indicates that BSP concentration in the liver cells must be many times that in the plasma.

Assuming a liver mass of approximately 250 g per 10 kg in the dog and 1,500 g in man, the average values for S of 25 mg per mg per 100 ml per 10 kg and 63 mg per mg per 100 ml in the two species would suggest that, with respect to plasma, the liver tends to concentrate BSP at least tenfold in the dog and fourfold in man. The range of normal values for S suggests that there is considerable variation in the biological mechanisms by which this concentration ratio is maintained from one subject to another and also, at least in the dog, from time to time in a given animal. This variation is greater than that observed in the Tm.

A high concentration ratio between liver and plasma might imply that some constituent of the liver cell has an affinity for BSP relatively greater than that of plasma protein. This would account for an ultimate relationship between liver and plasma concentrations but would certainly not explain the rapidity with which BSP, and other strongly protein-bound dyes such as rose bengal

(27) and indocyanine green (28), actually move across the liver cell membrane. More detailed understanding of the storage phenomenon must also take into account the possible role of BSP conjugation, since alteration of chemical structure might favor the accumulation of larger amounts of dye in the liver. However, as in the case of biliary secretion, there is no evidence at present to indicate whether or not conjugation plays an essential role in the storage process.

Relationship between storage and transport. In view of the anatomical relationship between the sinusoids, the liver cells, and the bile canaliculi, it might be postulated that plasma BSP is first taken up and stored in high concentration in liver cells from which it is then transported into the biliary tract (where it reaches even higher concentrations). However, it is also possible that there is a direct route of transport between plasma and bile and that the stored BSP is a more or less separate pool in equilibrium with the plasma. The latter possibility seems less likely when the observations with sodium dehydrocholate are considered. It was shown by Cantarow and Wirts (29) that the biliary excretion of BSP is markedly retarded when sodium dehydrocholate and BSP are administered simultaneously. In the present studies, by contrast, dehydrocholate was administered only after a large amount of BSP was already stored in the liver, and there was no reduction in BSP excretion. Thus, when hepatic removal of BSP from the plasma is partially blocked by dehydrocholate it becomes apparent that the dye can be secreted directly from hepatic parenchymal storage. Although the biliary secretion of BSP may depend upon the prior storage of dye within the liver cells, it is also obvious from the studies on patients with Dubin-Johnson syndrome that secretory transport can be markedly depressed even in the presence of an entirely normal storage capacity.

BSP Tm and S in liver disease. While a depression of BSP Tm was anticipated in all types of hepatic disease, a more nearly normal storage capacity might have been expected in the patients with biliary obstruction. It is not clear whether the reduced storage capacity in these patients is an indication of secondary parenchymal damage or whether it is due to the retention of specific substances which interfere with BSP storage. However, it is obvious that measurements of Tm and S could not be used clinically to distinguish between obstructive and hepatocellular jaundice.

Extrahepatic removal of BSP, which can be practically disregarded when hepatic function is normal (10, 30), should not be increased in patients with hepatic disease provided the BSP infusion rates are reduced sufficiently to avoid excessively high plasma concentrations. Nevertheless, the ratio of extrahepatic to total BSP removal is necessarily greater when hepatic function is impaired and this will affect the estimation of Tm or S or both (depending upon the behavior of the extrahepatic mechanisms). Hence the reduction in Tm and S in patients with liver disease must have been, if anything, even more marked than the values in Table III and in Figure 5 would indicate.

It is noteworthy that the estimated values of Tm exceeded 1 mg per minute in all pateints with total biliary obstruction. While this may be explained in part by extrahepatic BSP removal, it is difficult to reconcile this explanation with the consistently lower Tm values observed in patients with Dubin-Johnson syndrome (whose extrahepatic removal should be just as great). The possibility that there is some secretion of BSP into the obstructed biliary tree cannot be excluded.

The findings in patients with Dubin-Johnson syndrome support the prevailing concept (20) that this disorder involves a specific excretory defect without significant disturbance of other hepatocellular functions. The values of Tm and S observed in this syndrome would lead one to anticipate a sharp break in the BSP disappearance curve and a markedly reduced quantity of BSP in the bile as noted by Mandema, de Fraiture, Nieweg and Arends (31) following a single BSP injection. However, the regularly demonstrable relationship between hepatic BSP storage and plasma BSP concentration is inconsistent with the viewpoint, proposed by these authors, that hepatic storage capacity can be defined by an absolute maximal value. Patients with Dubin-Johnson syndrome, like normal subjects, appear to store BSP in the hepatic parenchyma in high concentration relative to plasma. In contrast to normal subjects, however, they are practically incapable of transporting BSP into the biliary tract.

SUMMARY

The rate of removal (R) of sulfobromophthalein sodium (BSP) from the blood is a function of *1*) the rate of secretion of dye into the bile and *2*) the rate of accumulation (or depletion) of dye in the hepatic parenchyma. BSP transport into the bile is a rate-limited process which becomes constant (Tm) when the plasma concentration of BSP is in excess of 3 mg per 100 ml. The accumulation of BSP in the hepatic parenchyma appears to proceed toward an equilibrium state in which the quantity of "stored" dye is proportional to BSP concentration in the surrounding plasma.

Extrahepatic tissue uptake is relatively small and may be considered negligible in defining BSP removal (R) in terms of active transport and storage as follows: $R = Tm + S \cdot (\Delta p/\Delta t)$, where $\Delta p/\Delta t$ is the change in plasma BSP concentration in milligrams per 100 ml per minute and S is the number of milligrams stored per milligram per 100 ml of plasma BSP concentration. The quantity "S" has been designated as the "relative storage capacity." The two unknowns, Tm and S, may be determined by the solution of simultaneous equations from values for R and $\Delta p/\Delta t$ obtained from the slopes of the plasma BSP concentration curves at different constant rates of BSP infusion.

Since S, which has the dimensions of volume, has proved to be several times larger than the volume of the liver itself, it may be inferred that BSP is concentrated in the liver cells. The linear correlation between R and $\Delta p/\Delta t$ at various infusion rates in the normal dogs or human subjects is consistent with constancy of both Tm and S within the range of BSP concentrations explored.

In six dogs, BSP Tm averaged 1.9 mg per minute per 10 kg body weight, and S was 25 mg per mg per 100 ml per 10 kg body weight. The maximal rate of biliary BSP excretion (measured colorimetrically) in the dog proved to be 87.5 ± SD 13.4 per cent of the value for Tm.

Direct transport of BSP from hepatic parenchymal storage into bile was demonstrated by the fact that excretory rate was maintained at a time when hepatic uptake from plasma was inhibited by sodium dehydrocholate (two dogs).

In 19 normal human subjects Tm was 8.6 ± SD 1.9 mg per minute, and S was 63 ± SD 25 mg per mg per 100 ml. Both values were diminished in patients with obstructive or hepatocellular jaundice. In three patients with Dubin-Johnson syndrome the value for Tm approached zero while the relative storage capacity was entirely normal.

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REFERENCES

1. Rosenthal, S. M., and White, E. C. Studies in hepatic function. VI. A. The pharmacological behavior of certain phthalein dyes. B. The value of selected pththalein compounds in the estimation of hepatic function. J. Pharmacol. exp. Ther. 1924, **24**, 265.
2. Wirts, C. W., Jr., and Cantarow, A. A study of the excretion of bromsulphthalein in the bile. Amer. J. dig. Dis. 1942, **9**, 101.
3. Brauer, R. W., and Pessotti, R. L. Hepatic uptake and biliary excretion of Bromsulphthalein in the dog. Amer. J. Physiol. 1950, **162**, 565.
4. Mendeloff, A. I., Kramer, P., Ingelfinger, F. J., and Bradley, S. E. Studies with bromsulfalein: II. Factors altering its disappearance from the blood after a single intravenous injection. Gastroenterology 1949, **13**, 222.
5. Graffin, A. L., and Bagley, E. H. Studies of hepatic structure and function by fluorescence microscopy. Johns Hopk. Hosp. Bull. 1952, **90**, 395.
6. Hanzon, V. Liver cell secretion under normal and pathologic conditions studied by fluorescence microscopy on living rats. Acta physiol. scand. 1952, **28**, suppl. 101.
7. Taplin, G. V., Meredith, O. M., Jr., and Kade, H. The radioactive (I^{131}-tagged) rose bengal uptake-excretion test for liver function using external gamma-ray scintillation counting techniques. J. Lab. clin. Med. 1955, **45**, 665.
8. Glaser, W., Gibbs, W. D., and Andrews, G. A. The mechanism of removal of rose bengal from the plasma of the rat. J. Lab. clin. Med. 1959, **54**, 556.
9. Combes, B., Wheeler, H. O., Childs, A. W., and Bradley, S. E. The mechanisms of Bromsulfalein removal from the blood. Trans. Ass. Amer. Phycns 1956, **69**, 276.
10. Wheeler, H. O., Epstein, R. M., Robinson, R. R., and Snell, E. S. Hepatic storage and excretion of sulfobromophthalein sodium in the dog. J. clin. Invest. 1960, **39**, 236.
11. Mason, M. F., Hawley, G., and Smith, A. Application of the saturation principle to the estimation of functional hepatic mass in normal dogs. Amer. J. Physiol. 1948, **152**, 42.

12. Lewis, A. E. Investigation of hepatic function by clearance techniques. Amer. J. Physiol. 1950, **163**, 54.
13. Taleisnik, S. Liver mass determination by bromsulphalein in partially hepatectomized rabbits. Gastroenterology 1955, **29**, 64.
14. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J. The estimation of hepatic blood flow in man. J. clin. Invest. 1945, **24**, 890.
15. Verschure, J. C. M. Clinical use of measurements of clearance and maximum capacity of the liver. Acta med. scand. 1952, **142**, 409.
16. Croxton, F. E., and Cowden, D. J. Applied General Statistics. New York, Prentice-Hall, 1946, p. 399.
17. Thomas, J. E. An improved cannula for gastric and intestinal fistulas. Proc. Soc. exp. Biol. (N. Y.) 1941, **46**, 260.
18. Cantarow, A., Wirts, C. W., Snape, W. J., and Miller, L. L. Excretion of bilirubin and Bromsulfalein in bile. Amer. J. Physiol. 1948, **154**, 211.
19. Lorber, S. H., Oppenheimer, M. J., Shay, H., Lynch, P. and Siplet, H. Enterohepatic circulation of bromsulphalein: Intraduodenal, intraportal, and intravenous dye administration in dogs. Amer. J. Physiol. 1953, **173**, 259.
20. Dubin, I. N. Chronic idiopathic jaundice. A review of fifty cases. Amer. J. Med. 1958, **24**, 268.
21. Krebs, J. S., and Brauer, R. W. Metabolism of sulfobromophthalein (BSP) in the rat. Amer. J. Physiol. 1958, **194**, 37.
22. Meltzer, J. I., Wheeler, H. O., and Cranston, W. I. Metabolism of sulfobromophthalein sodium (BSP) in dog and man. Proc. Soc. exp. Biol. (N. Y.) 1959, **100**, 174.
23. Combes, B. The biliary excretion of sulfobromophthalein sodium (BSP) in the rat as a conjugate of glycine and glutamic acid. J. clin. Invest. 1959, **38**, 1426.
24. Grodsky, G. M., Carbone, J. V., and Fanska, R. Identification of metabolites of sulfobromophthalein. J. clin. Invest. 1959, **38**, 1981.
25. Brauer, R. W., Pessotti, R. L., and Krebs, J. S. The distribution and excretion of S^{35}-labeled sulfobromophthalein-sodium administered to dogs by continuous infusion. J. clin. Invest. 1955, **34**, 35.
26. Wheeler, H. O., and Ramos, O. L. Determinants of the flow and composition of bile in the unanesthetized dog during constant infusions of sodium taurocholate. J. clin. Invest. 1960, **39**, 161.
27. Delprat, G. D., Jr. Studies on liver function. Rose bengal elimination from the blood as influenced by liver injury. Arch. intern. Med. 1923, **32**, 401.
28. Wheeler, H. O., Cranston, W. I., and Meltzer, J. I. Hepatic uptake and biliary excretion of indocyanine green in the dog. Proc. Soc. exp. Biol. (N. Y.) 1958, **99**, 11.
29. Cantarow, A., and Wirts, C. W., Jr. The effect of dog's bile, certain bile acids and India ink on bilirubinemia and the excretion of bromsulfalein. Amer. J. dig. Dis. 1943, **10**, 261.
30. Bradley, S. E., Clinical aspects of hepatic vascular physiology *in* Transactions of the Ninth Conference on Liver Injury. New York, Josiah Macy, Jr., Foundation, 1950, p. 71.
31. Mandema, E., de Fraiture, W. H., Nieweg, H. O., and Arends, A. Familial chronic idiopathic jaundice (Dubin-Sprinz disease), with a note on bromsulphalein metabolism in this disease. Amer. J. Med. 1960, **28**, 42.
32. Boothby, W. M., and Sandiford, R. B. Nomographic charts for the calculation of metabolic rate by the gasometer method. Bost. med. surg. J. 1921, **185**, 337.

THE DESTRUCTION OF RED CELLS BY ANTIBODIES IN MAN. III. QUANTITATIVE FACTORS INFLUENCING THE PATTERNS OF HEMOLYSIS *IN VIVO* *

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During the past several years it has become apparent through experimental studies with Cr^{51}-labeled red cells that injured red cells are removed from the circulation in either of two general ways. In the first, altered red cells are selectively trapped in the spleen. Such *splenic trapping* can be induced by coating the cell surface with adherent proteins, as with incomplete agglutinins (1, 2) or metallo-protein complexes (1, 3), or by increasing red cell thickness, as when red cells are made spherocytic by exposure to heat (4) or to lecithin (5). In addition, immature red cells (6, 7) and red cells injured by "oxidant" drugs, such as acetylphenylhydrazine (5, 8) are preferentially, although not so avidly, sequestered by the normal spleen. When relatively small amounts of Cr^{51}-labeled red cells are injected into man and the splenic filter remains "unsaturated," red cells coated by incomplete antibodies or rendered spherocytic *in vitro* are removed from the circulation by the spleen at a constant rate of approximately 3 ± 1 per cent per minute (2, 9), a rate which probably approaches the splenic blood flow.[1] In some instances red cells so trapped in the spleen may be released subsequently into the circulation (5).

In the second manner of red cell removal, more severe alterations of red cells are involved and the cells are sequestered diffusely throughout the reticuloendothelial system. Thus the liver, with its great mass, is the chief site of trapping. Such *hepatic trapping* ensues when red cells have been exposed to agglutinating antibodies (1, 2), complement-fixing antibodies (12, 13), agglutinating metallic cations (1, 3), sodium arsenate (4), and sterile incubation at 37° C for 24 hours or more (5). In general, these alterations involve either gross aggregation of red cells or a near-lytic injury to the cell membrane. When injected, small amounts of such cells are usually cleared rapidly, with half-disappearance rates of from 1.5 to 6 minutes. This presumably reflects the large blood flow of all the reticuloendothelial organs combined, among which the liver is foremost. In general, hepatic trapping of red cells kinetically resembles that of foreign particulate matter of many kinds. Although erythrophagocytosis may be provoked by some of the forms of red cell injury enumerated above, and although phagocytosis is often regarded as the essence of foreign particle clearance (14), phagocytosis is not visible during the initial stage in the hepatic trapping of red cells (15).

To what extent the contrasting pathways of red cell trapping outlined above are determined by qualitative, and to what extent by quantitative, factors is not certain. At least three purely quantitative factors are presumed to be involved: *1*) the quantity of altered red cells present; *2*) the extent of the alteration; *3*) the quantity of filtering (largely reticuloendothelial) tissue available (*2*). Although with respect to immune mechanisms, earlier studies had shown that the amount of antibody (anti-D) added to the appropriate (type D) red cells *in vitro* influenced the rate at which the

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[1] Although no accurate data exist as to the blood flow of the normal human spleen, it can be roughly computed from knowledge of the hemoglobin content and blood mixing time. Since the normal spleen contains about 7 g of hemoglobin (10), or approximately 1 per cent of that of circulating blood, and since, according to Harris, McAlister and Prankerd (11), splenic "mixing" of normal Cr^{51}-labeled red cells occurs within about 15 seconds, the normal splenic blood flow may be estimated to be approximately 1 per cent per 0.25 minute, or 4 per cent per minute.

affected cells were trapped by the spleen (*2*), it has not been ascertained whether quantitative differences in the extent of a given alteration might change the predominant pathway of red cell destruction from splenic to hepatic, or vice versa. At least two previous observations suggested, albeit inconclusively, that this might be the case. *1*) Whereas on injection into a normal subject, type D red cells sensitized *in vitro* with anti-D were selectively trapped in the spleen, type D red cells injected into immunized subjects whose plasma contained very high concentrations of anti-D were trapped to a moderate extent in the liver as well (*2*). *2*) Although the liver is the major site of sequestration when type B (or A) red cells are injected into subjects with detectable complete antibodies against such cells, at least some subjects with agammaglobulinemia (i.e., extreme hypogammaglobulinemia) sequester ABO-incompatible red cells in the spleen (*2*, 16).

There were inherent difficulties in the previous studies (*2*) of the quantitative effects of antibody added to red cells *in vitro* in terms of the subsequent fate of these cells *in vitro;* such studies involve a variable dissociation of antibody from the red cell after its injection into the circulation, the rate of this dissociation depending in part on the D antigen concentrations of the injected red cells and of the recipient's red cells. In order to explore quantitative factors influencing the effects of antibodies on red cells *in vivo,* the studies reported below were carried out in man and in rats.

METHODS

Techniques applicable to studies in man

Subjects. All studies relating to the effects of anti-B were carried out in a 56-year old woman with acquired agammaglobulinemia and less than 20 mg per 100 ml of gamma globulin in her serum. Previous studies (*2*) had shown that the patient, whose red cells were type A, possessed no anti-B which could be demonstrated *in vitro,* and that, as compared to their abrupt destruction in normal type A subjects, type B red cells were destroyed very slowly in the patient (half-survival time, 5 days), almost entirely in the spleen. Furthermore, after small weekly injections of type B red cells had been given, labeled type B red cells survived even longer (half-survival time, 22 days), suggesting ready exhaustion of a very small antibody pool. Relative to the studies conducted, this patient was regarded as possessing essentially no iso-antibody and as being suitable for passive immunization experiments.

Studies of the action of anti-D were conducted in one D-negative individual who had been specifically immunized against the D antigen at the Blood Grouping Laboratory, Boston, Mass., and in three elderly D-negative men who had no history of sensitization to the D antigen and whose sera contained no anti-D activity *in vitro.*

Techniques involving donor red cells and antisera. In all studies of anti-B action, type B red cells from the same individual were employed. Passive sensitization with anti-B was carried out by injecting aliquots taken from a single frozen specimen of serum [2] containing anti-B obtained from an individual specifically hyperimmunized against the B antigen. In high concentrations in the presence of fresh serum complement, this serum hemolyzed type B red cells; in saline it agglutinated a 5 per cent suspension of red cells to a titer of 1/200, and rendered the cells agglutinable in polyvinylpyrrolidone (PVP) to a titer of 1/800.

In all studies of anti-D action, type D red cells from the same individual were employed. Passive sensitization of the three D-negative subjects was accomplished as described below, by injecting aliquots taken from a single frozen specimen of plasma containing high concentrations of a nonagglutinating anti-D. This serum caused no agglutination of a 5 per cent suspension of type D red cells in saline at any concentration but rendered the cells agglutinable in PVP at antiserum dilutions of up to 1/16,000 and in Coombs serum at antiserum dilutions of up to 1/1,000. This anti-D serum was obtained from the same person who served as the actively immunized subject.

Techniques involving Cr^{51}. In each experiment approximately 25 ml of sterile whole donor blood was collected in siliconized screw-capped tubes containing 5 ml of acid citrate dextrose (ACD) solution. To each was added Cr^{51} of high specific activity (200 to 300 μc per μg) in the form of $Na_2Cr^{51}O_4$ [3] in amounts varying from 50 μc, in the patient subjected to multiple studies, to 200 μc in the subjects studied only once. After 30 minutes' gentle agitation at room temperature, the red cells were washed once in 10 to 15 volumes of sterile isotonic saline, were suspended in approximately 50 cent concentration in saline, a hematocrit reading was made, and an amount was injected which contained between 5 and 15 ml of packed red cells.

Peripheral blood from the injected subjects was obtained in a syringe containing one-tenth volume of 3 per cent sodium citrate. After mixing in test tubes, 3.0 ml samples were removed for the determination of whole blood radioactivity. The remainder of these blood specimens were centrifuged at approximately 2,000 G; the supernatant plasma was removed and was respun at 2,000 G before 3.0 ml samples were removed for measuring plasma radioactivity and plasma hemoglobin levels. Radioactivity of these materials was measured in a well-type scintillation counter and suitable correction factors

[2] Generously provided by Dr. A. R. Jones of the Blood Grouping Laboratory, Boston, Mass.

[3] Abbott Laboratories, Chicago, Ill.

were applied for dilution of whole blood and of plasma by the anticoagulant.

Body surface radioactivity over the heart, liver and spleen of each subject was measured periodically as previously described (17). At the conclusion of each experiment a complete body scan was conducted seeking high concentrations of radioactivity in other areas. None such was found.

Method of study in vivo. In each experiment involving passive immunization the subject was first injected with the sample of Cr^{51}-labeled, noncompatible red cells. For a period of 4 to 6 days the survival of these red cells and the subject's body surface radioactivity were followed. Then an amount of antiserum against the labeled cells, calculated to provide a desired ratio of antibody/red cells in the subject's circulation, was injected. During the subsequent several hours, numerous determinations of blood and corporeal radioactivity were made as illustrated in Figure 1. The acutely falling levels of red cell radioactivity after injection of antiserum were plotted on semilogarithmic paper, and the half-survival time was computed. The change in organ radioactivity was obtained by subtracting the relative radioactivity over each organ (17) just prior to antiserum injection from that measured after the cessation of the acute hemolytic process. The red cell trapping activity of the spleen relative to the liver (S/L) was then estimated by dividing the increment in splenic radioactivity by the increment in hepatic activity. An example of these calculations, as carried out in the experiment portrayed in Figure 1, is as follows:

$$\Delta S = S_a - S_b = 1.95–1.35 = 0.60$$
$$\Delta L = L_a - L_b = 1.80–0.63 = 1.17$$
$$S/L = \Delta S/\Delta L = 0.60/1.17 = 0.51$$

where ΔS and ΔL = increments in splenic and hepatic radioactivity, respectively, S_b and L_b = ratio of radioactivity spleen/precordium and liver/precordium, respectively, just before the injection of antiserum. S_a and L_a = ratio of radioactivity spleen/precordium and liver/precordium, respectively, after the cessation of the acute hemolytic process.

Techniques applicable to studies in rats

Subjects. The studies were made on large (400 to 500 g) males from a Bartonella-free strain (Charles River C. D.) of Sprague-Dawley rat.

Techniques involving donor red cells and antiserum. The rat red cells involved in each experiment were derived from fresh defibrinated blood obtained by cardiac puncture of normal animals of the same strain. Antiserum against rat red cells was obtained from a single adult male New Zealand rabbit which had received monthly intraperitoneal injections of saline-washed rat red cells for 8 months. Freshly drawn, this serum when incubated at 37° C with equal volumes of 5 per cent red cells in normal rat serum caused hemolysis to a titer of 1/100, agglutination to a titer of 1/100, and induced agglutinability in the presence of PVP to a titer of 1/8,000 (Table II). After heating for 2 hours at 56° C, the same antiserum in the presence of large volumes of fresh rat serum, caused hemolysis only when undiluted, agglutination to a titer of 1/100, and agglutinability in PVP to a titer of 1/8,000. All studies *in vivo* involved the use of aliquots taken from a single frozen specimen of this antiserum after it had been heated as described.

Techniques involving Cr^{51}. Rat red cells were labeled by adding 20 to 30 μc of Cr^{51} as $Na_2Cr^{51}O_4$ per ml of defibrinated rat blood, and agitating gently for 30 minutes at room temperature. The blood was then centrifuged, the serum removed, and the red cells made up to 50 per cent by volume in isotonic saline preparatory to the addition of antiserum (see below).

Radioactivity in the circulating blood of rats was assessed on blood obtained from small terminal incisions in the rats' tails. Two 0.1 ml samples were pipetted directly from the tail wound into test tubes suitable for use in a well-type scintillation counter. The first 0.1 ml sample was added to a tube containing 1.0 ml of heparinized water and was used to measure whole blood radioactivity. The second 0.1 ml sample was added to heparinized saline. This suspension was then centrifuged and the supernatant decanted into a second tube for the counting of plasma radioactivity. Red cell radioactivity was determined as the difference between whole blood and plasma radioactivity.

The radioactivity of rat livers, spleens, lungs, kidneys, and femurs was also determined in a well-type scintillation counter as described elsewhere (7).

Method of study in vivo. To the saline suspension of Cr^{51}-labeled rat red cells was added an equal volume of a predetermined dilution of antiserum. This mixture was incubated at 37° C with continuous gentle agitation for 1 hour, whereupon it was centrifuged, the plasma discarded, and the cells made up to a 50 per cent suspension in fresh rat serum for injection. Each rat was injected by tail vein with 0.5 ml of labeled, antibody-treated red cells, and thereafter serial blood specimens were withdrawn by tail puncture. At approximately 2.5 hours the animals were sacrificed, and the various organs were weighed and counted.

Peripheral blood radioactivity as a percentage of injected Cr^{51} was calculated from the product of the Cr^{51} concentration and the estimated blood volume divided by the total injected dose. The blood volume in animals of this strain and size was shown by previous studies with Cr^{51}-labeled normal red cells to be approximately 4.5 per cent of body weight. Organ radioactivity was calculated both as a percentage of injected Cr^{51} and as the concentration of Cr^{51} per wet weight.

Miscellaneous techniques

The presence of frank agglutination of red cells was determined by adding 0.1 ml of cell suspension to 10 ml of saline and examining microscopically a drop of the suspension. Increased red cell agglutinability was determined with PVP as described elsewhere (18). The presence of cell-adherent protein was determined by the technique of Coombs, Mourant and Race (19). Com-

TABLE I

Influence of antibody/antigen ratio on the destruction of Cr^{51}-labeled, type B red cells by anti-B in a passively immunized patient with agammaglobulinemia

Chronological order of experiments	Red cells injected		Antiserum injected			Effect of antiserum *in vivo*					Sites of red cell sequestration*		
						Red cell destruction			Maximum plasma Cr^{51}				
	Initial volume	Initial half-survival time	Days after red cell injection	Volume red cells present	Ratio antiserum/red cells			Rate	Per cent Cr^{51} injected	Peak time	ΔS	ΔL	S/L
	ml	*days*		*ml*		%	*ml*	*%/min*		*min*			
3	11.9	9	6	7.3	0.01	23	1.8	1.1	0		0.38	0.11	3.5
5	14.8	11	6	11.7	0.02	40	5.9	3.7	0		0.98	0.55	1.8
2	9.3	15	5	6.9	0.07	87	6.0	13.9	0.2		1.17	1.05	1.1
1	7.8	10	4	5.1	0.59	100	5.1	34.7	8.6	30	0.60	1.17	0.5
4	5.0	11	5	3.6	10.00	100	3.6	>50	100	2			

* As explained in the text, these figures are based on body surface radioactivity and are only relative, semiquantitative measurements, whereas the data from rats (Table V) are quantitative.

plement fixation was determined in the usual manner (20), but employing the same rabbit serum-rat red cell system in the complement assay.

Plasma hemoglobin levels were determined by the benzidine technique (21), using the same specimens employed for measuring plasma radioactivity.

RESULTS

Anti-B. Five studies were carried out in the patient with agammaglobulinemia, and the data are presented in Table I. The chronological sequence of these experiments, involving differing ratios of antiserum/red cells, was randomized. During the four to six days prior to the injection of anti-B serum, the Cr^{51}-labeled type B red cells injected into the patient were destroyed at a relatively slow, steady rate, with estimated half-survival times of from 9 to 15 days. At the time of injection of the anti-serum, from 6.9 to 11.7 ml of red cells remained, except in the experiment involving a large dose of antiserum in which a smaller volume of cells was deliberately employed. When a very small amount of antiserum was employed so that the estimated antiserum-red cell ratio *in vivo* was only 0.01, there followed a rapid disappearance of 23 per cent of the circulating red cell radioactivity, at the rate of 1.1 per cent of the injected cells per minute. There was no subsequent rise in plasma hemoglobin or Cr^{51}. A small, definite rise in splenic radioactivity ensued, associated with a barely perceptible rise in hepatic activity. A slightly larger volume of antiserum (0.02 ml per ml type B red cells), caused an acute fall of 40 per cent of the type B red cells at the rate of 3.7 per cent per minute. Again there was no rise in plasma levels of hemoglobin or Cr^{51}. A sharp rise in splenic radioactivity and a simultaneous, somewhat smaller increase in hepatic activity were observed. The injection of 0.07 ml of antiserum per ml type B red cells eliminated 87 per cent of the Cr^{51}-labeled red cells from the circulation, at the rate of 13.9 per cent per minute. This was associated with a very low level of Cr^{51} in the plasma with no definite point of maximal activity. In this experiment both spleen and liver radioactivity increased sharply and to about the same extent. The administration of 0.5 ml of antiserum per ml of type B red cells eliminated 100 per cent of the red cell radioactivity from the patient's circulation, at the rate of 34.7 per cent per minute. In this experiment (Figure 1) there followed a rise in plasma hemoglobin and of Cr^{51}, the latter reaching a maximal level of 8.6 per cent of the injected dose 30 minutes after the injection of the antiserum. The rise in hepatic radioactivity was about double that over the spleen. Within two minutes of the injection of a large volume of antiserum, 10 ml per ml of type B red cells, all of the injected Cr^{51} was found to be in the plasma, as was over 90 per cent of the injected red cell hemoglobin. Whereas none of the other experiments elicited symptoms from the patient, this hemolyzing dose of antiserum [4] caused facial flushing to occur, beginning two minutes after injection and lasting four or five minutes. About seven minutes after the antiserum injection, pain in the groins, thighs, and low back appeared, and persisted for four or five minutes.

[4] This association between intravascular hemolysis and immediate reactions to incompatible red cells has been described elsewhere (5).

The relation between the ratio of anti-B serum/type B red cells and the relative trapping of type B red cell by spleen and liver is portrayed graphically in Figure 2. The largest and frankly hemolyzing dose of antiserum is not included in this figure. For comparison the ratio of spleen/liver radioactivity after the injection of type B cells into a type A subject, with approximately the same concentration of agglutinating anti-B antibodies but lacking anti-B hemolysins, is included. In this instance the subject's plasma volume was substituted for the volume of anti-B-containing serum.

The effects of the anti-B serum upon type B red cells *in vitro* is compared with its effects *in vivo* in Table II. The suspending and diluting medium employed *in vitro* was the agammaglobulinemic patient's serum. Agglutination in serum,

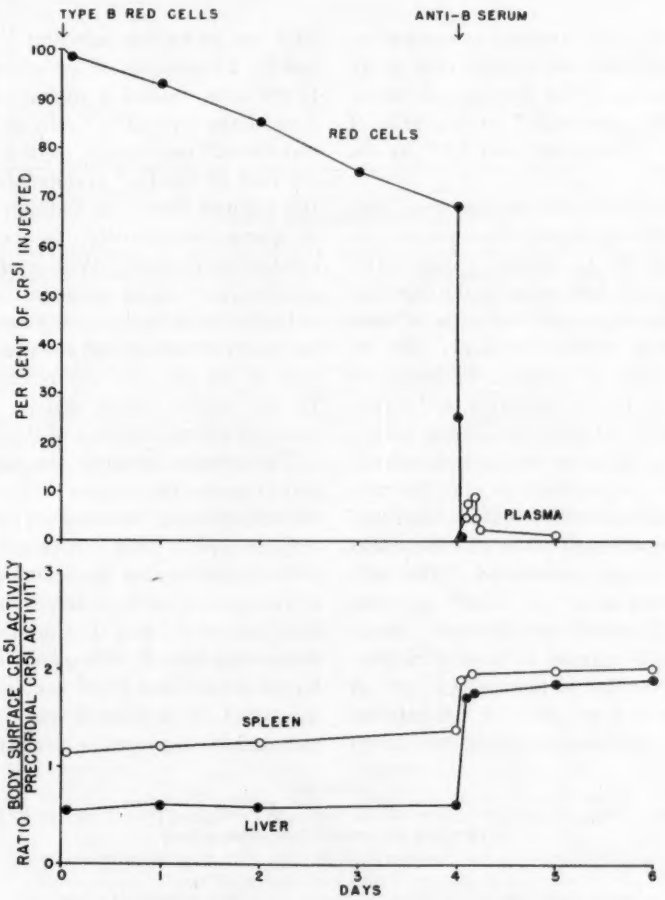

FIG. 1. EFFECT OF ANTI-B ON SEQUESTRATION OF TYPE B CELLS IN A TYPE A PATIENT WITH AGAMMAGLOBULINEMIA. After the injection of Cr^{51}-labeled type B red cells, splenic radioactivity became somewhat elevated and increased slightly during the four days before antiserum injection. When antiserum was injected, note the abrupt fall in circulating Cr^{51}-labeled red cells followed by a slightly delayed rise in plasma radioactivity (upper portion of figure). With the injection of anti-B, splenic radioactivity and, to an even greater extent, hepatic radioactivity sharply increased and then remained stable (lower portion).

TABLE II

Comparison of the effects of anti-B on type B red cells in vitro and in vivo

Ratio anti-B serum / type B red cells	Effects *in vitro*					Effects *in vivo*		
		Agglutination					Sequestration	
	Hemolysis	In serum	In plasma	PVP	Coombs serum	Intravascular hemolysis	Spleen	Liver
0.01	0	0	0	0	0	0	2+	±
0.02	0	0	±	1+	±	0	3+ to 4+	2+
0.07	0	1+	1+ to 2+	3+	2+	0	4+	4+
0.59	0	2+	2+ to 3+	4+	4+	±	2+	4+
10.00	3+					4+		

in plasma, and in PVP was assessed by suspending 1 volume of packed, antibody-treated cells in 10 volumes of the medium. The presence of "intravascular hemolysis" was judged on the basis of the appearance of hemoglobin and Cr^{51} in the plasma (Table I).

Anti-D. Three individual studies were conducted on three different, D-negative subjects, not immunized against the D antigen (Table III). During the four to six days prior to the injection of anti-D, Cr^{51}-labeled type D red cells in these subjects appeared to survive normally, with no abnormal accumulation of radioactivity anywhere in the body. At the time of injection of the antiserum, approximately 10 ml of circulating labeled red cells remained. When a relatively small volume of antiserum was injected, so that the ratio of antiserum/type D red cells was 0.14, there was a delay of almost 30 minutes before an exponential decline of radioactivity commenced. The cells were then eliminated at a rate of 0.9 per cent per minute until all were gone. This was associated with a marked increase in splenic radioactivity and almost no rise in hepatic activity. A maximum of only 1.3 per cent of the injected Cr^{51} appeared in the plasma almost two hours after the antiserum injection. A larger dose of anti-D, 2.7 volumes of antiserum per ml of type D red cells, caused a slightly more rapid clearance of the type D red cells so that 1.3 per cent was cleared per minute, with a maximum of 2.2 per cent of the Cr^{51} accumulating in the plasma 108 minutes later. In addition to a marked rise in splenic radioactivity, the hepatic activity increased moderately. With a still larger dose of anti-D, 13.34 ml of antiserum per ml of type D red cells, the red cell clearance was at a rate of 1.8 per cent per minute and the maximal plasma Cr^{51} level of 4.0 per cent was reached in 95 minutes. In this study, hepatic activity was still higher, reaching almost half that of the spleen.

The relation between the ratio of volume of anti-D serum/the volume of type D red cells and the distribution between spleen and liver of trapped type D cells is depicted in Figure 2. In addition two studies are included in which 10 ml of the same type D red cells were injected into the donor of the anti-D serum employed. In both studies the type D cells were cleared without delay at a rate of 3.8 per cent per minute, and a maximum of between 5 and 7 per cent of the radioactivity appeared in the plasma at about one

TABLE III

Influence of antibody-antigen ratio on the destruction of Cr^{51}-labeled type D red cells by anti-D in passively immunized D-negative subjects

Red cells injected		Antiserum injected			Effect of antiserum *in vivo*							
					Red cell destruction			Maximum plasma Cr^{51}		Sites of red cell sequestration		
Initial volume	Initial half-survival time	Days after red cell injection	Volume red cells present	Ratio antiserum/red cells			Rate	Per cent Cr^{51} injected	Peak time	ΔS	ΔL	S/L
ml	*days*		*ml*		%	*ml*	*%/min*		*min*			
11.2	30	6	9.8	0.14	100	9.8	0.9	1.3	114	5.00	0.10	50
11.5	28	4	10.4	2.70	100	10.4	1.3	2.2	108	3.78	1.07	3.5
10.5	25	4	9.0	13.34	100	9.0	1.8	4.0	95	4.6	2.2	2.1

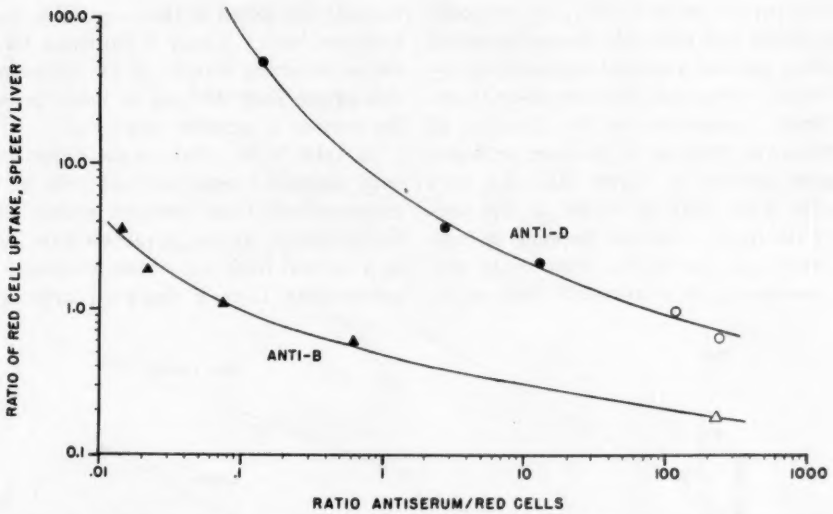

FIG. 2. QUANTITATIVE COMPARISON OF THE ABILITY OF ANTI-B AND ANTI-D ANTIBODIES TO CAUSE SPLENIC OR HEPATIC SEQUESTRATION OF RED CELLS. In a patient with agammaglobulinemia given extremely small amounts of anti-B serum (black triangles) the relative trapping of Cr^{51}-labeled type B red cells by spleen and liver resembled that produced by very large (several hundred times as much) amounts of anti-D serum with type D cells (black circles). The distribution of type B red cells injected into a type A recipient whose serum contained non-hemolyzing autogenous antibodies and an anti-B agglutinin titer comparable to that of the anti-B serum employed is depicted by the white triangles. The distribution of type D red cells injected on two different occasions into the D-negative donor of the anti-D serum employed above, is represented by the white circles. A log-log plot has been used.

hour. In the first study the hepatic Cr^{51} uptake slightly exceeded that of the spleen (S/P = 0.90); in the other the S/P ratio was only 0.65. One of these studies was included in a previous report (2).

The effects of the anti-D serum used upon the type D red cells employed in these studies *in vitro* and *in vivo* are presented in Table IV. Normal serum was used as a suspending and diluting medium in all titrations.

Rabbit antibodies against rat red cells. The effect of adding to rat red cells various amounts of rabbit antiserum developed against rat red cells, upon their subsequent survival *in vivo,* is presented in Table V. In none except the highest of the concentrations employed was there significant intravascular hemolysis, as judged by the uniformly low levels of radioactivity in the plasma and kidneys (15). The survival of the labeled red cells was curtailed in direct proportion to the amount of antibody employed. With relatively small amounts of antibody (0.17 to 0.33 ml rab-

TABLE IV

Comparison of the effects of anti-D on type D red cells in vitro and in vivo

Ratio $\frac{\text{anti-D serum}}{\text{type D red cells}}$	Effects *in vitro*					Effects *in vivo*		
		Agglutination				Intravascular hemolysis	Sequestration	
	Hemolysis	In serum	In plasma	PVP	Coombs serum		Spleen	Liver
0.01	0	0	1+	3+	1+		Not studied	
0.14	0	0	2+	4+	4+	0	4+	0–±
2.70	0	0	2+	4+	4+	0	4+	2+
13.34	0	0	2+	4+	4+	0	4+	3+

bit antiserum per ml rat red cells), the red cells were incompletely and relatively slowly destroyed and the spleen was the dominant sequestering organ. At higher antiserum concentrations, however, in linear proportion to the quantity of antibody employed, trapping by the liver predominated (upper portion of Figure 3). As portrayed in the lower part of Figure 3, this preemption by the liver of red cell trapping at high antibody doses was due to the tendency of this relatively massive organ to approach (but not to exceed) the spleen in trapping ability on a gram-for-gram basis. Figure 4 illustrates the fact that the sequestering activity of the spleen relative to that of the liver declined in linear proportion to the amount of antibody employed.

In Table V the effects of the rabbit serum antibody employed upon rat red cells *in vitro* are compared with those observed *in vivo*. Analogous to the human studies, titrations were carried out in a normal fresh rat serum medium. It is apparent from Table V that even large amounts of

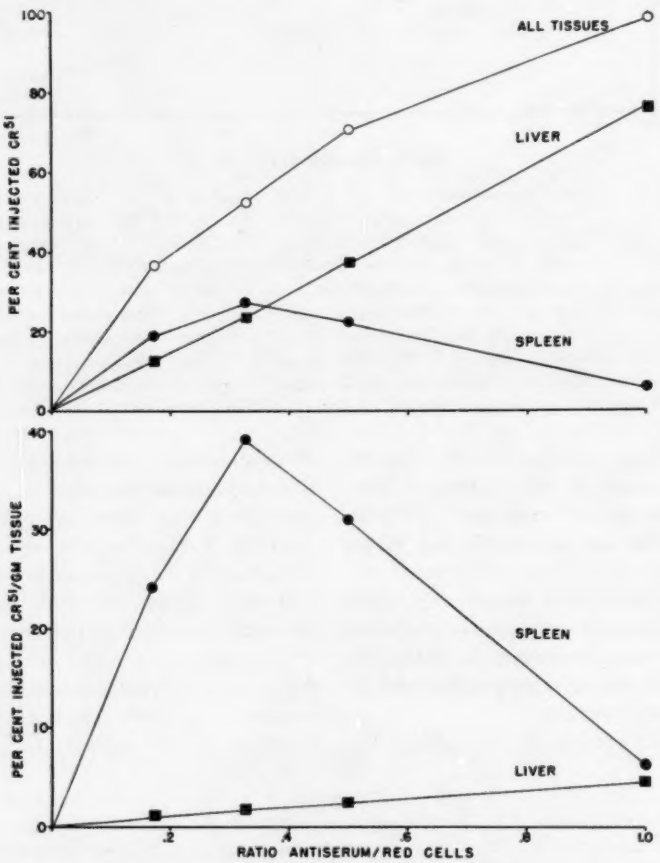

FIG. 3. THE EFFECT OF VARIOUS ANTIBODY/ANTIGEN RATIOS ON THE SEQUESTRATION *IN VIVO* OF RAT RED CELLS PREVIOUSLY TREATED *IN VITRO* WITH RABBIT ANTI-RAT RED CELL SERUM. In the upper portion of the figure it is evident that the total sequestration of Cr^{51}-labeled red cells ("all tissues") increased in proportion to the antiserum/red cell ratio. The total splenic uptake of Cr^{51} reached a maximum at an antiserum/red cell ratio of 0.33, when its ability to trap red cells was over 20 times that of the liver, on a gram-for-gram basis (lower portion). With larger amounts of antibody, the spleen and liver approximated each other in trapping ability per gram tissue (lower portion), and therefore the total liver uptake predominated.

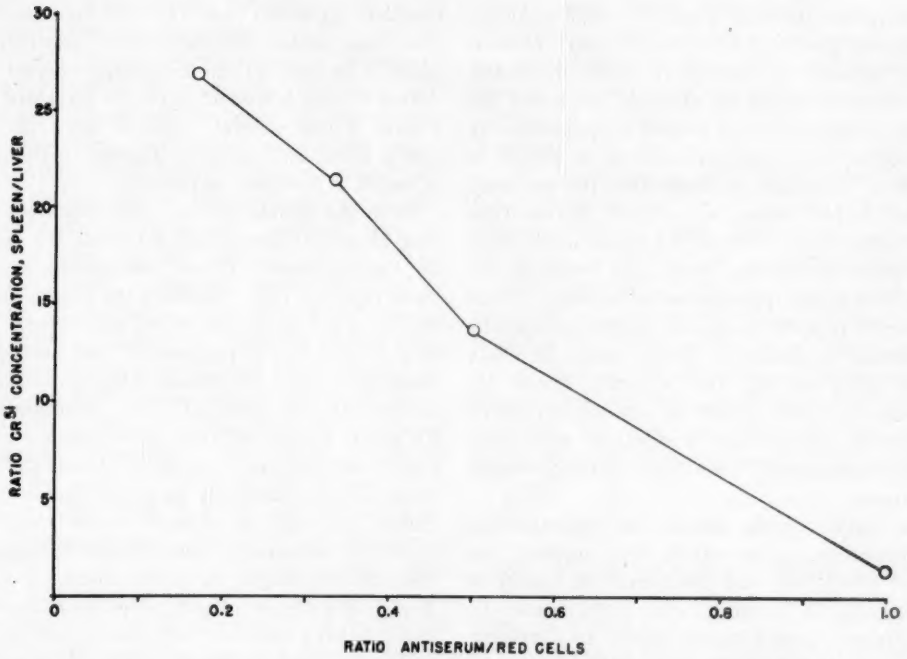

FIG. 4. RELATIONSHIP BETWEEN ANTIBODY/ANTIGEN RATIOS AND THE SPLEEN/LIVER RATIOS IN RATS. Replotting the data from the lower portion of Figure 3 reveals that the extent of sequestration of antibody-treated, Cr^{51}-labeled, red cells by the spleen relative to the liver bears an inverse relationship to the amount of antibody present.

this antibody failed to provoke any red cell sequestration in the lungs or femur. The very low levels of radioactivity in the lungs correlated with the levels of activity remaining in the peripheral blood. Only when there was some evidence of intravascular hemolysis, as indicated by plasma radioactivity, was there any appreciable kidney radioactivity.

DISCUSSION

In general these studies support previous observations (1, 2) to the effect that antibodies which bind to red cells without agglutinating or hemolyzing them may cause a predominant trapping of the cells by the spleen. It is clear, however, that this pattern is affected by quantitative factors. Thus in the experiments with anti-D,

TABLE V

The effects of rabbit anti-rat red cell serum in vitro and in vivo

No. of rats	Ratio rabbit antiserum / rat red cells	Effects *in vitro*			Effects *in vivo*								
					Red cell destruction			Cr^{51} uptake					
			Agglutination					Spleen		Liver			
		Hemolysis	In serum	In PVP	Per cent destroyed in 2 hrs	Rate	Intravascular hemolysis	Total		Total		Lungs Total	Femur Total	Kidney Total
						%/min		%/g	%	%/g	%	%	%	%
2	0.17	0	0	3+	37.0	1.1	0	24.1	18.8	0.9	12.4	0.6	0.1	0.5
2	0.33	0	1+	4+	52.5	1.9	0	38.9	27.2	1.8	24.4	0.5	0.1	0.5
8	0.50	0	2+	4+	70.4	4.3	0	30.9	22.4	2.3	37.6	0.3	0.1	0.4
2	1.0	±	3+	4+	98.7	34.6	±	6.3	6.1	4.8	76.5	0.1	0.1	1.3
2	2.0	1+	4+	4+	97.0	34.6	2+	10.8	12.2	2.9	49.6	0.3	0.2	3.7

an incomplete antibody, relatively small amounts of antibody produced a slow and quite selective splenic pattern of destruction, while increasing the amount of circulating antibody quickened the rate of sequestration and evoked sequestration by the liver as well. By extrapolation, as shown in Figure 2, it appears probable that the tendency of some highly immunized subjects to trap type D red cells in the liver as well as, or even somewhat better than, in the spleen may reflect an extreme limit of this quantitative relationship. Thus the anti-D type of incomplete agglutinin may be considered to possess a certain range of effect on red cells, varying from a purely splenic sequestration, when small to moderately large amounts of antibody are involved, to a partially hepatic mechanism when very large amounts are present.

The studies with anti-B, an agglutinating, complement-fixing, potentially lytic antibody, indicated that it, too, had a spectrum of activity *in vivo*, ranging in its effects, when given in nonlytic doses, from a predominantly splenic to a strongly hepatic pattern of sequestration. With this antibody, however, splenic sequestration was less active than with anti-D, occurred over a rather narrow range of antibody/antigen proportion, and was selective only at barely injurious antibody concentrations which were, indeed, undetectable by their effects *in vitro*.[5] With small to moderate amounts of such antibody, a tendency for hepatic trapping to occur is manifest and predominates over a wide range of antibody-antigen ratios, until actual intravascular hemolysis is encountered. As shown in Figure 2 this relationship can be extrapolated to include, at the extreme, data on the actively immunized subject whose plasma contained a relative infinity of nonhemolytic agglutinating anti-B antibody. The spectrum of action *in vivo* of the potentially lytic and agglutinating antibody (anti-B), may, through quantitative variation, overlap the spectrum of action of the incomplete agglutinin (anti-D). On the other hand, if one may assume that the antibody concentration (anti-D or anti-B) in these hyperimmune sera differs by only a fewfold, it can be estimated from Figure 2 that several hundred times as much anti-D is required to mimic a given, small amount of anti-B in its effects *in vivo*.

With the volume of red cells employed, trapping by anti-D always led to a small, but detectable, rise in plasma Cr^{51} and hemoglobin, as previously reported (2). Similarly the hepatic sequestration of 5.1 ml of type B red cells by anti-B led to a delayed rise in plasma Cr^{51} and hemoglobin, reaching a peak 30 minutes after antiserum injection. On the other hand, the sequestration of 5.9 and 6.0 ml of red cells by smaller amounts of anti-B antibody in Experiments 5 and 2, respectively, caused essentially no rise in plasma hemoglobin, although the rate of sequestration was rapid (as compared to anti-D) and the sequestration was permanent. A similar absence of plasma hemoglobin despite rapid, permanent sequestration, has been observed with autogenous red cells previously incubated at 37° C for 48 hours (5). Therefore, contrary to previous deductions (2), it may be that any significant rise in plasma hemoglobin during immune hemolysis may reflect the action of complement or complement-like intravascular substances.

The studies in rats involved the use of a heterologous antiserum, which after heating contained only a small amount of lytic antibody (even in the presence of large volumes of fresh rat serum), a moderate concentration of agglutinin, and a high concentration of an incomplete agglutinin. The pattern of destruction *in vivo* of red cells pretreated with such antiserum *in vitro* was markedly influenced by the quantity of the antiserum employed. Thus, small amounts of antibody, sufficient to destroy only partially the treated red cells, caused a slow (1.1 per cent per minute), predominantly splenic trapping. As larger and larger amounts of antibody were employed, the liver, in direct linear proportion, assumed the greater role. Thus, at a serum/red cell ratio of 1.0, the liver accounted for about 80 per cent of the sequestered red cells, and, as in the anti-B experiments, a red cell clearance rate of about 35 per cent per minute was attained. It is apparent from an examination of Figure 3 and Table

[5] Presumably the lowest anti-B level was provided by the patient with agammaglobulinemia herself, prior to the present studies, when, despite lack of detectable antibody *in vitro*, she sequestered type B cells slowly, over several days, in the spleen. That splenic sequestration of normal red cells on an immune basis may occur despite the absence of incompatibility *in vitro* has been previously reported (22) and confirmed (23, 24).

IV that the liver assumed the major role in red cell destruction by pre-emption rather than by simply receiving the overflow from a "saturated" spleen. Finally, with large, lytic volumes of antiserum actual intravascular hemolysis was evident and radioactivity, presumably radioactive hemoglobin, accumulated for the first time in the kidney. This spectrum of hemolytic activity of rabbit antibody in rats closely paralleled that of anti-B in man (compare Tables I and V). Thus, in these studies in rats the same antibody, or mixture of antibodies, was capable of causing three distinct patterns of hemolysis: *1*) a slow, incomplete trapping of red cells in the spleen; *2*) a rapid, complete sequestration, chiefly in the liver; and *3*) an intravascular lysis with deposition of hemoglobin throughout the reticuloendothelial system and with spillage into the kidneys. Although previous studies in dogs had indicated that red cells coarsely agglutinated by metallic cations may lodge in the lungs (*2*), in the present studies the lungs were devoid of red cell trapping activity, regardless of the intensity of agglutination of the injected red cells; nor was there evidence of hemoglobin uptake by the lungs. Furthermore, pulmonary sequestration of red cells, apart from extravasations into the alveoli, as in essential pulmonary hemosiderosis (*25*), has not been observed experimentally or clinically in man. Thus it appears that the reticuloendothelial tissue of the lungs seldom, if ever, participates in hemolytic mechanisms.

The mechanisms by which antibody-injured red cells are sequestered are still in doubt. There is reasonably good, but necessarily indirect, evidence that incomplete agglutinins destroy red cells by rendering them agglutinable in the presence of fibrinogen and other anisometric macromolecules *in vivo* (*2*, 18), which in turn leads to their low pressure filtration (*26*) by the spleen. Although it has been postulated that frankly agglutinating antibodies, such as anti-B, cause hepatic trapping by causing large, firm agglutinates which are also filterable by the relatively large liver sinusoids (*2*), the extensive, comparative studies of human iso-antibodies by Cutbush and Mollison (12, 13, *27*) indicate that those antibodies which bind complement, without producing frank agglutination *in vitro*, also cause a rapid (probably hepatic) pattern of red cell destruction. Thus, in the present studies, some hepatic sequestration was observed at antibody concentrations which caused little or no agglutination *in vitro*. However, hepatic uptake of red cells was not pronounced until agglutinating amounts of antibody were employed. It appears that the liver may partially participate in red cell trapping, in a "spleen-like" fashion, either in the splenectomized subject (*2*) or in the presence of a vast excess of incomplete agglutinin, but that a purely hepatic pattern of destruction usually commences with, or is coincident with, actual red cell agglutination demonstrable *in vitro*. Whether the prime mechanism of hepatic sequestration involves a simple physical filtration of lattice-forming red cell agglutinates (*26*), possibly involving "uncoated" red cells (*28*), or a surface adherence to fixed macrophages, is yet to be determined.

SUMMARY

The influence of antibody/antigen proportions on the kinetics of red cell destruction were studied in man and in rats.

The effects of anti-B iso-antibody were studied by passively immunizing an agammaglobulinemic patient with various amounts of anti-B serum and observing the pattern of destruction of a given amount of type B red cells. A spectrum of effects was observed, ranging from a slow partial trapping by the spleen with small doses to a brisk hepatic trapping with large doses, while still larger doses caused intravascular hemolysis.

The effects of incomplete anti-D iso-antibody were studied by passively immunizing each of several D-negative subjects with various amounts of anti-D serum and observing their effect upon given amounts of type D red cells. Although small and moderate amounts of antibody caused a mainly splenic pattern of destruction, large amounts caused a moderate hepatic uptake and were similar in their effects to very small amounts of anti-B antibody.

Studies on rats employing a rabbit antibody against rat red cells indicated a spectrum of action similar to that of anti-B in man: a slow, incomplete sequestration by the spleen occurred when small amounts of antibody were employed; a rapid, largely hepatic sequestration occurred with larger amounts; and with still larger amounts, intravascular lysis was seen, with hemoglobin

deposition in the liver, spleen and kidney. The lungs and bone marrow did not participate in the sequestration process at any dose of antibody.

Although the kinetics of red cell destruction *in vivo* by complete and by incomplete agglutinins ordinarily differ, the differences are modified by purely quantitative factors.

REFERENCES

1. Jandl, J. H. Sequestration by the spleen of red cells sensitized with incomplete antibody and with metallo-protein complexes (abstract). J. clin. Invest. 1955, **34**, 912.
2. Jandl, J. H., Jones, A. R., and Castle, W. B. The destruction of red cells by antibodies in man. I. Observations on the sequestration and lysis of red cells altered by immune mechanisms. J. clin. Invest. 1957, **36**, 1428.
3. Jandl, J. H., and Simmons, R. L. The agglutination and sensitization of red cells by metallic cations: Interactions between multivalent metals and the red-cell membrane. Brit. J. Haemat. 1957, **3**, 19.
4. Harris, I. M., McAlister, J. M., and Prankerd, T. A. J. The relationship of abnormal red cells to the normal spleen. Clin. Sci. 1957, **16**, 223.
5. Jandl, J. H., and Tomlinson, A. S. The destruction of red cells by antibodies in man. II. Pyrogenic, leukocytic and dermal responses to immune hemolysis. J. clin. Invest. 1958, **37**, 1202.
6. Berendes, M. The proportion of reticulocytes in the erythrocytes of the spleen as compared with those of circulating blood, with special reference to hemolytic states. Blood 1959, **14**, 558.
7. Jandl, J. H. The agglutination and sequestration of immature red cells. J. Lab. clin. Med. 1960, **55**, 663.
8. Cruz, W. O. Acetylphenylhydrazine anemia. 1. The mechanism of erythrocyte destruction and regeneration. Amer. J. med. Sci. 1941, **202**, 781.
9. Hughes-Jones, N. C., Mollison, P. L., and Veall, N. Removal of incompatible red cells by the spleen. Brit. J. Haemat. 1957, **3**, 125.
10. Motulsky, A. G., Casserd, F., Giblett, E. R., Broun, G. O., Jr., and Finch, C. A. Anemia and the spleen. New Engl. J. Med. 1958, **259**, 1164.
11. Harris, I. M., McAlister, J., and Prankerd, T. A. J. Splenomegaly and the circulating red cell. Brit. J. Haemat. 1958, **4**, 97.
12. Cutbush, M., and Mollison, P. L. Relation between characteristics of blood-group antibodies *in vitro* and associated patterns of red-cell destruction *in vivo*. Brit. J. Haemat. 1958, **4**, 115.
13. Mollison, P. L. Blood-group antibodies and red-cell destruction. Brit. med. J. 1959, **2**, 1123.
14. Biozzi, G., Benacerraf, B., and Halpern, B. N. Quantitative study of the granulopectic activity of the reticulo-endothelial system. II. A study of the kinetics of the granulopectic activity of the R. E. S. in relation to the dose of carbon injected. Relationship between the weight of the organs and their activity. Brit. J. exp. Path. 1953, **34**, 441.
15. Kaplan, M. E., Schwartz, E., Weiss, L., and Jandl, J. H. Unpublished observations.
16. Chaplin, H., Jr. Studies on the survival of incompatible cells in patients with hypogammaglobulinemia. Blood 1959, **14**, 24.
17. Jandl, J. H., Greenberg, M. S., Yonemoto, R. H., and Castle, W. B. Clinical determination of the sites of red cell sequestration in hemolytic anemias. J. clin. Invest. 1956, **35**, 842.
18. Jandl, J. H., and Castle, W. B. Agglutination of sensitized red cells by large anisometric molecules. J. Lab. clin. Med. 1956, **47**, 669.
19. Coombs, R. R. A., Mourant, A. E., and Race, R. R. A new test for the detection of weak and "incomplete" Rh-agglutinins. Brit. J. exp. Path. 1945, **26**, 255.
20. Kabat, E. A., and Mayer, M. M. Experimental Immunochemistry. Springfield, Ill., Charles C Thomas, 1948.
21. Ham, T. H. A Syllabus of Laboratory Examination in Clinical Diagnosis. Cambridge, Mass., Harvard University Press, 1950.
22. Jandl, J. H., and Greenberg, M. S. The selective destruction of transfused "compatible" normal red cells in two patients with splenomegaly. J. Lab. clin. Med. 1957, **49**, 233.
23. Fudenberg, H., and Allen, F. H., Jr. Transfusion reactions in the absence of demonstrable incompatibility. New Engl. J. Med. 1957, **256**, 1180.
24. Adner, P. L., and Sjölin, S. Unexpected blood group incompatibility, revealed by ^{51}Cr-labelled red cells. Scand. J. clin. Lab. Invest. 1957, **9**, 265.
25. Jonsson, B., Vahlquist, B., and Agner, K. Essential hemosiderosis. Blood 1951, **6**, 665.
26. Jandl, J. H. Sequestration of reticulocytes and of abnormal red cells by filtration at low pressures (abstract). J. clin. Invest. 1958, **37**, 905.
27. Mollison, P. L., and Cutbush, M. Use of isotope-labelled red cells to demonstrate incompatibility *in vivo*. Lancet 1955, **1**, 1290.
28. Jones, A. R., and Kaneb, L. A new property of iso-agglutinins of the ABO blood group system. Blood 1959, **14**, 1094.

IMMUNOASSAY OF ENDOGENOUS PLASMA INSULIN IN MAN

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For years investigators have sought an assay for insulin which would combine virtually absolute specificity with a high degree of sensitivity, sufficiently exquisite for measurement of the minute insulin concentrations usually present in the circulation. Methods in use recently depend on the ability of insulin to exert an effect on the metabolism of glucose *in vivo* or in excised muscle or adipose tissue. Thus, the insulin concentration in plasma has been estimated: *a*) from the degree of hypoglycemia produced in hypophysectomized, adrenalectomized, alloxan-diabetic rats (1); *b*) from the augmentation of glucose uptake by isolated rat hemidiaphragm (2); or *c*) from the increased oxidation of glucose-1-C^{14} by the rat epididymal fat pad (3). Since there have been reports indicating the presence, in plasma, of inhibitors of insulin action (4) and of non-insulin substances capable of inducing an insulin-like effect (5, 6), these procedures, while yielding interesting information regarding the effects of various plasmas on glucose metabolism in tissues, are of doubtful specificity for the measurement of insulin per se (5).

Recently it has been shown (7, 8) that insulins from various species (pork, beef, horse and sheep) show quantitative differences in reaction and cross reaction with antisera obtained from human subjects treated with commercial insulin preparations (beef, pork insulin mixtures). An immunoassay method for beef insulin has been reported in which the insulin content is determined from the degree of competitive inhibition which the insulin offers to the binding of beef insulin-I^{131} by human antisera (9–12). Although human insulin reacts with human antibeef, pork insulin antiserum and displaces beef insulin-I^{131} by competitive inhibition (7, 8, 10), the reaction is too weak to permit measurement of the low insulin concentrations present in human plasma (7, 8, 11–13). In preliminary communications we have reported that the competitive inhibition by human insulin of binding of crystalline beef insulin-I^{131} to guinea pig antibeef insulin antibodies is sufficiently marked to permit measurement of plasma insulin in man (11, 12, 14), and to be capable of detecting as little as a fraction of a microunit of human insulin (12, 14). Preliminary data on insulin concentrations in man before and after glucose loading have been reported (12, 14, 15). The present communication describes in detail the methods employed in the immunoassay of endogenous insulin in the plasma of man, and reports plasma insulin concentrations during glucose tolerance tests in nondiabetic and in early diabetic subjects and plasma insulin concentrations in subjects with functioning islet cell tumors or leucine-sensitive hypoglycemia.

METHODS

Immunization of guinea pigs. Guinea pigs were injected subcutaneously at 1 to 4 week intervals with 5 to 10 units of either protamine zinc beef insulin (Squibb) or commercial regular beef insulin (Squibb) emulsified with mannide mono-oleate. Insulin-binding antibodies were detected in all animals after 2 to 3 injections. The antiserum employed in the present study (GP 49, serum 6-25-59) was obtained from a guinea pig immunized with protamine zinc beef insulin without adjuvant and was selected for its relatively high antibody concentration and other suitable characteristics described below.

Preparation of insulin-I^{131}. Because of the desirability of keeping the concentration of added insulin-I^{131} as low as possible and yet assuring an adequate counting rate, it is necessary to prepare the insulin-I^{131} with a high specific activity. The lots of insulin-I^{131} employed in this study had specific activities of 75 to 300 mc per mg at the time of use. The preparation of such highly labeled preparations entails difficulties not encountered when the specific activity is very much lower. The Newerly modification (16) of the Pressman-Eisen method (17) was used for labeling with several further modifications designed to increase specific activity and to minimize damage to the insulin from irradiation and other causes. To approximately 0.3 ml chloroform in a 50 ml separatory funnel are added in turn, 0.2 ml of 2.5 N HCl, 20 μl of 10^{-3} M KI, 30 to 80 mc I^{131} (as iodide) and 1 drop of 1 M $NaNO_2$. Immediately after addition of the last reagent, the funnel is stoppered to prevent loss of I^{131} into the atmosphere and is shaken vigorously for 2 to 3 minutes. The chloroform layer (bottom) is then drawn

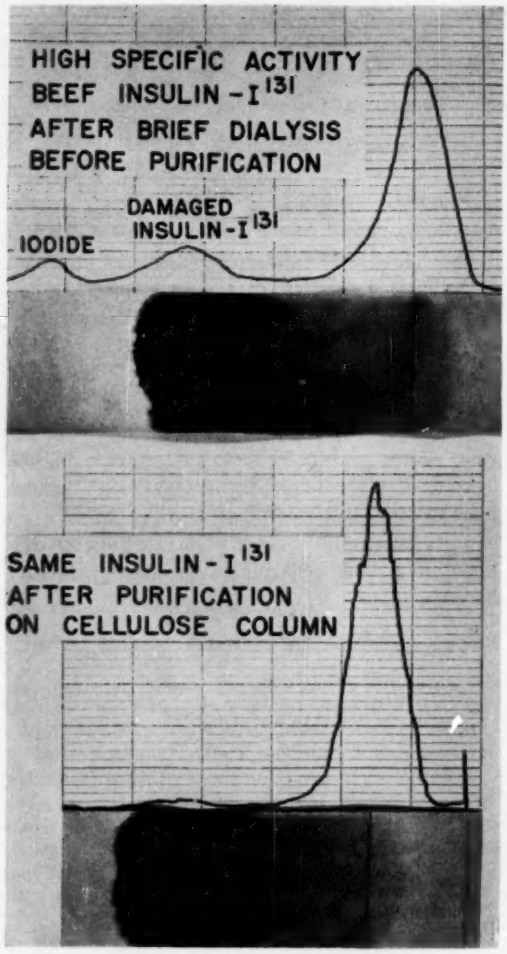

FIG. 1. PURIFICATION OF DAMAGED INSULIN-I^{131} BY CELLULOSE COLUMN ADSORPTION. *Top:* Chromato-electrophoretograms of beef insulin-I^{131}, with specific activity about 300 mc per mg, after 30 minutes' dialysis. *Bottom:* Same preparation after elution from cellulose column with control (nonimmune) plasma. At significantly lower specific activities the preparations appear as in the bottom figure *without* purification.

into a test tube beneath a layer of a few drops of water (to prevent loss of I^{131} into the air) and assayed for radioactivity in any low-sensitivity counting device. If much less than one-half of the starting radioactivity has been extracted, a second extraction with 0.2 to 0.3 ml chloroform is performed. The total amount of chloroform should be kept as small as possible to facilitate the subsequent extraction of iodine into the aqueous protein solution. The volume of the aqueous phase in the separatory funnel also should be kept small to favor the initial extraction of iodine into the chloroform. The chloroform-iodine mixture is added to 0.5 ml of 0.2 M borate buffer, pH 8, containing 20 μg of crystalline beef insulin in a 50 ml centrifuge tube, which provides for a broad interface between the two phases. The tube is shaken briskly but not violently for not more than 2 to 3 minutes following which an additional 1.0 ml borate buffer is mixed into the contents. A barely visible flocculate appears occasionally and should be allowed to settle, whereupon the top 0.5 ml (one-third of total) of the water phase is quickly removed and dialyzed against 2 L of distilled water.[1] Owing to the high concentration of radioactivity and low concentration of protein, the insulin is very susceptible to radiation damage (18, 19); therefore, exposure to I^{131} at this stage should be as brief as possible, not more than 5 to 10 minutes elapsing between addition of I^{131} to the insulin and the start of dialysis. Of the total radioactivity in the dialysis bag, approximately 65 to 80 per cent represents unbound I^{131} which is reduced to less than 1 per cent of the I^{131} bound to insulin after 2 hours of dialysis. Between 20 and 60 per cent of the insulin-I^{131} is adsorbed to the dialysis membrane during this time so that the procedure yields approximately 3 to 5 μg insulin labeled with about 1.0 to 2.0 mc I^{131}. Considerable sacrifices in total yield are made to expedite the procuring of a highly labeled preparation which usually contains no more than 4 to 6 per cent damaged components. We have the impression that the addition of 10^{-2} M KI or phenol (as radical scavengers) to the dialyzing solution may help to minimize radiation damage, but this has been difficult to establish since other factors are also responsible for damage to the protein during the procedure. Distilled water is used in the last dialysis following which 1 drop of human serum albumin (250 mg per ml) is added to the insulin-I^{131} solution to prevent losses of labeled insulin by adsorption to glassware (20, 21) and to minimize any further irradiation damage (18, 19). Solutions are kept frozen when not in use.

If the insulin-I^{131} solution is surveyed for radioactivity at completion of dialysis, the specific activity of the insulin-I^{131} may be estimated approximately. If the yield of labeled insulin has been sufficient to produce a specific activity in excess of 150 mc per mg, it can be anticipated that damage will be significantly in excess of 4 to 6 per cent, and at 300 mc per mg may be as great as 15 to 18 per cent. It is then necessary to effect partial purification of the insulin-I^{131}. Since the damaged components do not adsorb to paper but are observed to migrate with serum proteins on paper strip chromatography or electrophoresis (22), it is possible to use a cellulose column for the purification procedure as follows: The dialyzed insulin-I^{131} solution is added to 0.1 ml control (nonimmune) serum and the mixture is then passed through a column packed

[1] Removal of unbound iodide^{131} by anionic exchange resins is usually unsatisfactory because much of the insulin-I^{131} at this low concentration is lost by adsorption to the resin.

with a cellulose powder [2] about 1 ml in volume following which the column is washed 3 or 4 times with 1 ml of veronal buffer, 0.1 ionic strength. Most of the damaged components pass through the column with the serum while the undamaged insulin remains adsorbed to the cellulose in the column and can now be eluted slowly with undiluted control serum or plasma. Usually 3 to 4 eluates (each 0.5 ml of plasma) are collected and diluted immediately 1:20 to 1:100 with veronal buffer containing 0.025 per cent serum albumin to prevent further damage to the insulin by the concentrated plasma. Although the elution of insulin-I^{131} from the column is far from complete, adequate amounts are obtained for almost any number of insulin assays. Most of the damaged fraction is removed by this procedure (Figure 1).

Principles of immunoassay. The basis of the technique resides in the ability of human insulin to react strongly with the insulin-binding antibodies present in guinea pig antibeef insulin serum (11, 12, 14), and by so doing, to inhibit competitively the binding of crystalline beef insulin-I^{131} to antibody. The assay of human insulin in unknown solutions is accomplished by comparison with known concentrations of human insulin. The use of I^{131}-labeled animal insulin as a tracer is necessitated by the lack of a crystalline preparation of human insulin.

The determination of antibody-bound insulin-I^{131} and free insulin-I^{131} by paper chromato-electrophoresis has been described previously (22). Briefly, the separation of antibody-bound insulin from unbound insulin in plasma results from the adsorption of all free insulin (when present in amounts less than 1 to 5 μg) to the paper at the site of application ("origin"), while the antibody-bound insulin migrates toward the anode with the inter-β-γ-globulins. Thus, in the presence of insulin-I^{131} there appear two separate peaks of radioactivity; measurement of the areas beneath the two peaks (by planimetry) yields the relative proportion of bound insulin-I^{131} (migrating with serum globulins) and free insulin-I^{131} (remaining at origin). The ratio of bound insulin-I^{131} to free insulin-I^{131} (B/F) is a function of the concentration of insulin-binding antibodies, of both insulin concentrations, and of the characteristic kinetic and thermodynamic constants for the reactions between the insulins and the particular antiserum (23). Selection of an antiserum for purposes of this assay is determined primarily by the desirability of obtaining a relatively marked decrease in B/F ratio with small increments in the concentration of human insulin. Although the antibody concentration is of only secondary importance, it should be high enough to permit at least 1:100 dilution of the antiserum (preferably 1:1,000 dilution or greater). On the basis of preliminary tests the antiserum is diluted appropriately to yield an initial B/F ratio between 2 and 4 for tracer beef insulin-I^{131} alone, in the absence of added human insulin. Provided that the amount of the beef insulin-I^{131} used is truly a tracer quantity, the initial B/F ratio is inversely proportional to the dilution factor (23). In the presence of human insulin, the B/F ratio decreases progressively with increase in insulin concentration; with sensitive antisera the B/F ratio is reduced by about 50 per cent in the presence of 15 μU per ml human insulin.

Standard curves. Two preparations of human insulin were employed as standards. The first ("Tietze human insulin")[3] is reported (24) to have a potency of 1.8 U per mg crude preparation; the second ("Fisher human insulin"),[3] was assayed at 6.8 U per mg in 1956 (25), but it was believed that the activity of the latter preparation might have decreased slightly since its initial preparation (25). A tentative value of 6 U per mg for the Fisher insulin was assigned. However, since a value as low as 22 U per mg could be placed on a crystalline sample of the latter preparation (25), whereas the Tietze insulin was assayed relative to a standard of 27 to 29 U per mg, we have regarded the Tietze crude insulin preparation as $1.8/28 \times 100 = 6.45$ per cent pure insulin by weight, and the Fisher insulin powder preparation as $6/22 \times 100 = 28.2$ per cent pure insulin by weight. When compared on this basis, no consistent differences in potencies of the two preparations were observed in the immunoassay procedure and the value of 6 U per mg for the Fisher preparation was accepted as the correct value. Since the Fisher preparation is the more highly purified, it was employed as standard in most of the studies.

All dilutions of insulin and antiserum are prepared in 0.1 ionic strength veronal buffer containing 0.25 per cent human serum albumin to prevent adsorption of reactants to glassware. (There is no detectable insulin in commercial supplies of human serum albumin.) Standard solutions each contain identical concentrations of tracer beef insulin-I^{131} (about 0.05 to 0.15 mμg per ml but differing in different runs) and antiserum, but varying concentrations of human insulin ranging from 0.05 to 5.0 mμg per ml (calculated as "pure" human insulin). The antiserum is added last in all cases. Mixtures are refrigerated at 4° C for 4 days. These conditions provide sufficient time to reach equilibrium between bound and free insulin. The mixtures are then subjected to chromato-electrophoresis (22) in a cold room at 4° C (Whatman 3 MM paper, veronal buffer, 0.1 ionic strength, pH 8.6, constant voltage 20 to 25 v per cm, cover of apparatus open), which produces a satisfactory separation of the peaks of bound and free insulin-I^{131} in about 1 to 1.5 hours. Earlier immunoassays (10) were performed after prolonged incubation at 37° C. However, it has since been shown (23) that the standard free energy change of the reaction in the direction of antigen-antibody complex formation is increased considerably at 4° C, which results in an approximately twofold greater slope in the B/F versus insulin concentration curves at low insulin concentrations. Just prior to chromato-

[2] Genuine Whatman Cellulose Powder, W & R Balston Ltd., England.

[3] We are greatly indebted to Dr. F. Tietze of the National Institutes of Health and Dr. A. M. Fisher of the Connaught Laboratories, Toronto, Canada, for these preparations.

electrophoresis, control (nonimmune) guinea pig plasma is added to the mixtures to prevent trailing of antibody-bound insulin on the paper strips, since the very low concentrations of serum proteins in these mixtures are insufficient in themselves to prevent adsorption of the serum proteins (including antibody) to the paper.

The chromato-electrophoretograms are developed until the albumin band has moved about 2.5 to 3 inches from the origin, which, under the conditions employed here, usually takes about an hour. The peak of antibody-bound insulin-I^{131} moves about 2.25 inches under these conditions. The use of several large boxes, each with a capacity for 16 strips, makes it possible to run 250 to 300 strips a day. After drying, the strips are assayed for radioactivity in an automatic strip counter (Figure 2A). A "standard curve" is obtained by plotting the B/F ratio as a function of the concentration of added human insulin (Figure 2B) after correction for damaged components of insulin-I^{131}. From 3 to 6 per cent of the insulin-I^{131} was damaged after final preparation of the lots employed in this study. These damaged components migrate nonspecifically with the serum proteins, primarily with the α-globulins (22), and are demonstrably not available for binding by antibody. The short run chromato-electrophoresis does not resolve the serum proteins well enough to distinguish between antibody-bound insulin-I^{131} and damaged insulin-I^{131} so that the damaged fraction is determined by using either control (nonimmune) plasma, or antiserum whose binding capacity for undamaged insulin-I^{131} is completely saturated with beef insulin. Since the antiserum used here has a maximal beef insulin-binding capacity of about 1 mμg per ml at the dilutions employed, it has been general practice to include one or more samples made up with 1 to 4 μg per ml beef insulin for the purpose of determining the damaged fraction. Damaged insulin-I^{131} migrating with serum proteins is corrected for by subtracting the fraction damaged from the total area in the chromato-electrophoretogram. The area under the free insulin peak is then divided by the corrected total area to yield the fraction of free insulin. The fraction "bound insulin" is then 1.00 minus the fraction "free insulin."

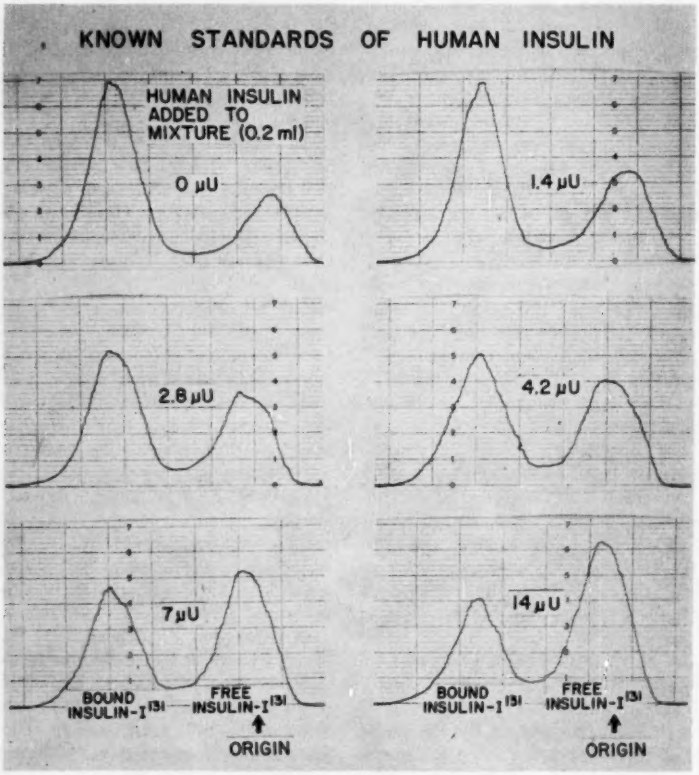

FIG. 2. A: RADIOCHROMATO-ELECTROPHORETOGRAMS OF ANTISERUM, INSULIN MIXTURES. Mixtures contained the same concentrations of guinea pig antibeef insulin serum and beef insulin-I^{131} but varying concentrations of human insulin as indicated.

It is evident that variation in the volumes of solution applied to the paper strips is of no consequence. Generally 100 to 200 μl is applied, the larger volume permitting use of a smaller quantity of tracer beef insulin-I^{131} for the same counting rate.

Assay of insulin in plasma. Mixtures containing unknown samples are prepared at the same time and in the same way as are standard solutions except that the unknown sample is substituted for the human insulin. Plasma insulin is best determined in a 1:10 final dilution unless the insulin concentration is unusually high; then a 1:20 or 1:40 dilution may be used. Mixtures may be made up to any desired volume. However, since only 100 to 200 μl is applied to the paper strips, it is convenient to prepare all mixtures in 0.2 or 0.5 ml volumes containing 20 or 50 μl of plasma, respectively.

Since insulin may be damaged by plasma during incubation (22), an effect which is more marked in concentrated plasma than in diluted plasma, and at 37° rather than at 4° C,[4] it is advisable to run a control mixture with unknown plasma but without antiserum to correct for "incubation damage." However, at 1:10 dilution of plasma after 4 days at 4° C, incubation damage amounts only to 0 to 3 per cent, an observation which contributed to the selection of these conditions. Therefore, only a negligible additional correction for damage is required in the plasma samples.

The insulin concentration in each plasma sample is determined from the standard curve by referring to the insulin concentration which corresponds to the corrected B/F ratio observed in the plasma sample (10–12, 14, 15).

Subjects for glucose tolerance tests. Subjects were chosen at random from patients sent to the general laboratory for glucose tolerance tests and from known diabetic and apparently nondiabetic patients on the wards of the Veterans Administration Hospital, Bronx, N. Y. Patients who had *ever* been treated with insulin were excluded from this study in order to obviate effects of antibodies in their own serum (22). Other than the exception noted below, subjects were classified as diabetic or nondiabetic on the basis of the following criteria applied to the 2-hour blood sugar curve following oral ingestion of 100 g of glucose: *diabetic*—a peak blood sugar concentration of 180 mg per 100 ml or greater, and a 2-hour blood sugar concentration of 120 mg per 100 ml or greater; *nondiabetic*—a peak blood sugar concentration not exceeding 160 mg per 100 ml, and 2-hour level no more than 120 mg per 100 ml. One subject with marginal ulcer and a dumping syndrome, with a blood sugar concentration of 286 mg per 100 ml at 0.5 hour falling to 134 mg per 100 ml at 1 hour and 44 mg per 100 ml at 2 hours, is included in this group. Because of the exclusion of insulin-treated patients, only mild or early maturity-onset diabetes is represented in the diabetic group. Subjects who did not qualify by these criteria for either group are considered in an *"undetermined status."* The criteria employed are modified from those suggested by Fajans and Conn (26) and are designed to eliminate questionable cases from diabetic and nondiabetic categories.

[4] For this reason plasma is separated in a refrigerated centrifuge immediately after withdrawal of blood and is used immediately or kept frozen until used in order to minimize loss of the endogenous insulin present.

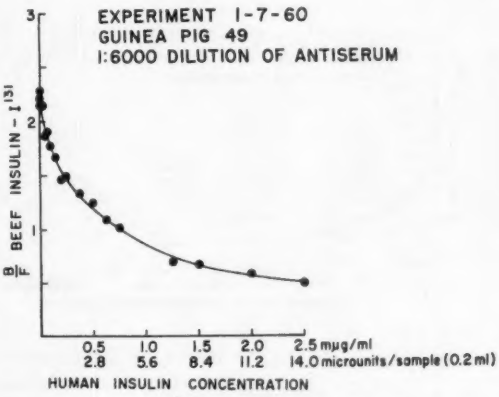

FIG. 2. B: RATIO, $\frac{\text{BOUND INSULIN-I}^{131}}{\text{FREE INSULIN-I}^{131}}$, AS A FUNCTION OF THE CONCENTRATION OF ADDED HUMAN INSULIN. The ratios were obtained from the complete series of radiochromato-electrophoretograms, a few of which are shown in Figure 2A.

All subjects were to have fasted for 14 hours prior to the glucose tolerance test, but from the fasting blood sugar concentration in one subject (Ri) it is suspected that this restriction was not observed in his case. All subjects were to have consumed a diet containing at least 300 g carbohydrate per day for 3 days preceding the glucose tolerance test, but there is no assurance that this regimen was followed in all cases. Blood samples were obtained in the fasting state immediately before, and 0.5 hour, 1 hour and 2 hours following glucose feeding. In a small group of cases an additional 50 g glucose was administered at 1.5, 2 and 2.5 hours, and blood collections were continued to 3 hours.

Blood sugar determinations were determined according to the method of Somogyi (27).

RESULTS

Standard curves. Several representative standard curves are shown in Figure 3. The amount of insulin-I^{131} employed as tracer varied somewhat from experiment to experiment. In the experiments shown in Figure 4, the effects of Tietze and Fisher insulins are compared with each other and with the effect of crystalline beef insulin. As in other experiments no significant differences between the two human insulin preparations were observed. Since 100 to 200 μl of solution was as-

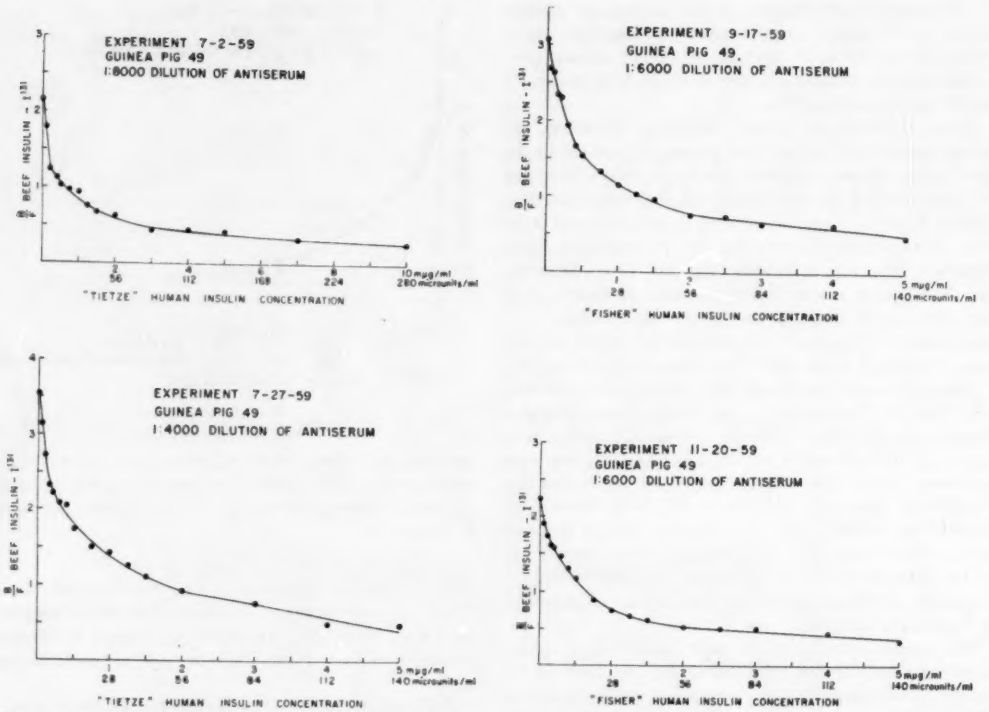

FIG. 3. STANDARD CURVES: B/F (BEEF INSULIN-I^{131}) RATIO AS A FUNCTION OF THE CONCENTRATION OF TIETZE OR FISHER HUMAN INSULIN.

sayed, less than 1 μU of human insulin was readily detectable with this antiserum. At low insulin concentrations, random variations in B/F produce only small errors in the absolute quantity of insulin but the percentage error is high; conversely, at high insulin concentrations the absolute error is likely to be higher but the percentage error lower. By increasing the dilution of the antiserum, the entire concentration range is easily scaled down by a factor of 2 or 3 and the limit of sensitivity increased to about 0.1 to 0.2 μU of insulin. However, the conditions employed are suitable for determination over the 50- to 100-fold range of insulin concentrations ordinarily encountered in man.

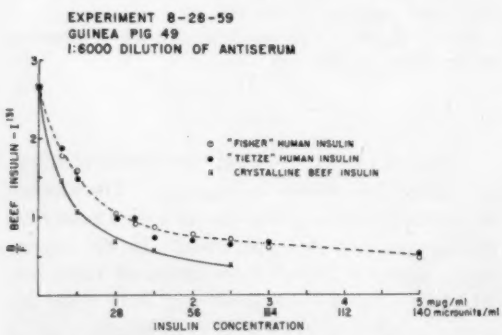

FIG. 4. COMPARISON OF THE EFFECTIVENESS OF VARIOUS CONCENTRATIONS OF TIETZE AND FISHER HUMAN INSULINS AND CRYSTALLINE BEEF INSULIN IN REDUCING THE B/F RATIO FOR BEEF INSULIN-I^{131}.

It is evident that beef insulin reacts about two to four times more strongly (depending on the insulin concentration employed) with the guinea pig antibeef insulin serum than does human insulin (Figure 4). Other guinea pig antisera to beef insulin have shown even greater differences in reaction of beef insulin and human insulin. On this account *beef insulin cannot be used as a standard for the assay of human insulin in the guinea pig antibeef insulin system.* Because of the differences in reactivity of human and beef insulin, differences in the specific activity of the beef insulin-I^{131} preparations result in different

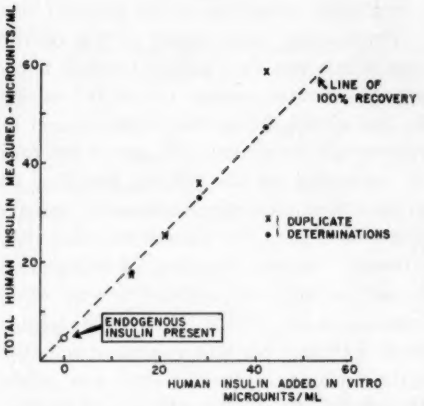

FIG. 5. A: RECOVERY OF HUMAN INSULIN ADDED *IN VITRO* TO A FASTING PLASMA SAMPLE. Endogenous insulin concentration in undiluted plasma was 48 μU per ml. All assays were performed in 1:10 dilution of plasma.

initial B/F values and somewhat differently shaped curves even at the same dilution of antiserum if approximately the same radioactivity (and therefore different amounts of beef insulin) is used. These differences could be abolished if each lot of beef insulin-I^{131} were assayed for its beef insulin concentration and if the same amount of beef insulin were employed, independent of its content of radioactivity. However, it is more expedient to include a standard curve with human insulin for each run of unknowns. When 250 or more unknown samples have been run in a single experiment, an added set of 15 to 16 standard solutions is a negligible addition.

TABLE I

*Effect of cysteine on endogenous plasma insulin**

Subj.	Plasma sample	Insulin concentration: Original plasma before incubation and dialysis	Control sample incubated and dialyzed without cysteine	Sample incubated with cysteine and dialyzed
		$\mu U/ml$	$\mu U/ml$	$\mu U/ml$
Yo.	1 hr	324	238	14
Un.	1 hr	337	216	0

* See text for conditions of experiments.

Recovery of added human insulin and effect of plasma dilution. The virtually quantitative recovery of human insulin added to plasma *in vitro* (Figure 5A) indicates that the plasma has neither an inhibitory nor an augmentative effect and this conclusion is confirmed by the proportionate decrease in measured insulin concentration when the plasma is diluted over a large range (Figure 5B).

Effect of cysteine and cellulose on endogenous plasma insulin. Since insulin is destroyed by incubation with cysteine at alkaline pH and is adsorbed by powdered cellulose, the effects of these agents on endogenous insulin were tested. Plasmas of relatively high insulin concentration were incubated at 37° C with 0.02 M cysteine at pH 8 for 1.5 hours and then dialyzed against normal saline for 3 hours to remove the cysteine. Aliquots of the same serum samples were treated

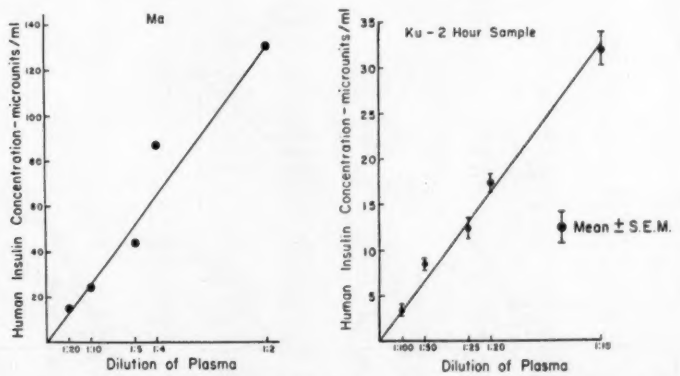

FIG. 5. B: EFFECT OF DILUTION OF PLASMA ON MEASURED CONCENTRATION OF ENDOGENOUS PLASMA INSULIN. Four replicate determinations were made for each point in the experiment on the right.

TABLE II

Cellulose adsorption of beef insulin-I^{131} and endogenous human plasma insulin

Subj.	Plasma	Per cent adsorbed by cellulose column: Beef insulin-I^{131}	Per cent adsorbed by cellulose column: Endogenous human insulin
	ml		
Y.	0.1	69	64
	0.2	49	44
U.	0.1	71	88
E.	0.1	81	84

similarly except that cysteine was omitted. Although incubation and dialysis alone led to a 26 to 36 per cent loss in endogenous insulin concentration in the control samples, cysteine was almost completely effective in destroying the endogenous insulin (Table I). In simultaneous experiments insulin-I^{131} was found to be virtually completely destroyed under these conditions as determined by paper chromato-electrophoresis.

To evaluate cellulose adsorption of endogenous insulin, a minute amount of tracer beef insulin-I^{131}, negligible compared to the amounts used in the immunoassay, was added to 0.1 or 0.2 ml plasma which was then passed through a packed powdered cellulose column (about 0.5 ml in volume) and eluted, by suction, with veronal buffer to recover all the plasma. Assay of the radioactivity remaining on the column and that eluted from the column, and immunoassay of endogenous insulin eluted from the column revealed that approximately the same fractions of endogenous insulin and added beef insulin-I^{131} were adsorbed by the cellulose (Table II). The larger the amount of plasma per unit volume of cellulose the less the fraction of insulin that was adsorbed. With significantly smaller amounts of plasma virtually all insulin-I^{131} and endogenous insulin are adsorbed, but the insulin concentrations then become unmeasurable. Only negligible fractions of albumin-I^{131} and γ-globulin-I^{131} are adsorbed by cellulose under these conditions.

Insulin concentrations in early maturity-onset diabetic and control subjects. The average fasting insulin concentrations tended to be only slightly

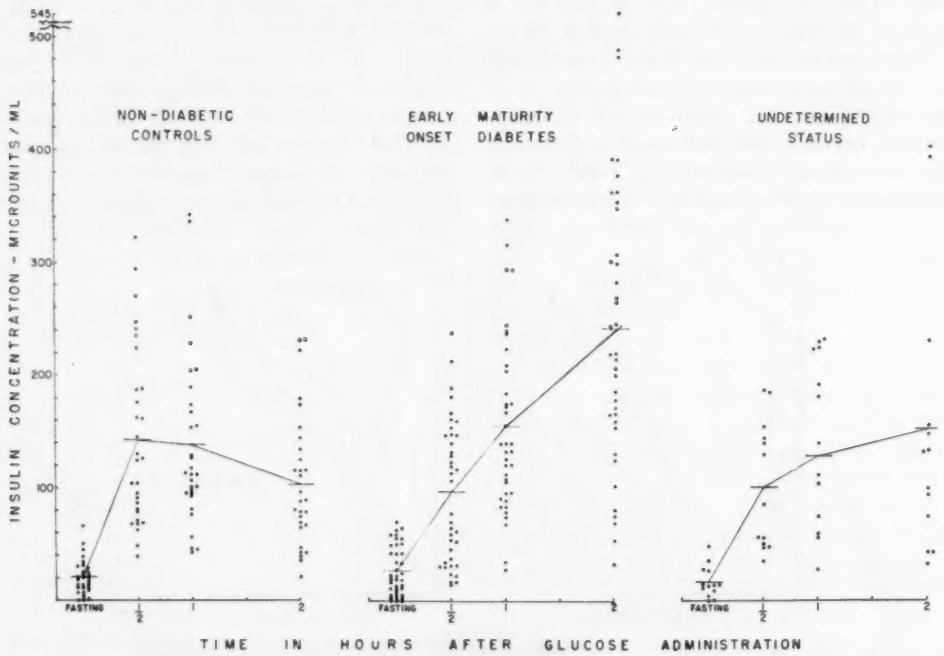

FIG. 6. PLASMA INSULIN CONCENTRATIONS DURING STANDARD 100 G (P.O.) GLUCOSE TOLERANCE TEST IN VARIOUS GROUPS OF SUBJECTS.

TABLE III

Blood sugar and plasma insulin concentrations during a standard 100 g oral glucose tolerance test

Subj.	Blood sugars						Plasma insulin concentrations					
	F	0.5 hr	1 hr	2 hrs	3 hrs	4 hrs	F	0.5 hr	1 hr	2 hrs	3 hrs	4 hrs
	mg/100 ml						*μU/ml*					
A. *Nondiabetic controls*												
La.	87	122	78	91			31	162	112	89		
Pa.	73	109	68	77			25	270	154	155		
Hu. J.	85	120	113	113			7	103	95	116		
Wh.	78	110	85	88			66	294	168	174		
Don.	88	108	135	115			45	67	98	39		
St.	89	120	104	93			13	95	46	43		
Kan.	83	113	103	83			28	62	229	117		
Ra.	91	131	135	90			20	176	128	112		
Jo.	92	113	113	80			25	124	118	21		
Wa.	98	128	147	118			20	68	93	75		
Dor.	90	140	148	118			31	81	126	90		
Ei.	83	100	70	95			50	235	101	79		
Cor.	110	133	133	115			21	130	155	145		
Cal.	91	117	147	119			7	39	76	47		
Te.	83	143	155	73			17	78	190	126		
Ru.	83	103	113	98			3	145	204	222		
Dam.	90	118	120	110			18	187	205	180		
Kas.	98	150	135	108			11	48	112	65		
Kr.	98	115	125	120			2	71	95	117		
Him.	93	140	120	108			22	322	252	232		
Hu. J. J.	95	128	110	105			9	126	56			
Dan.	83	115	73	100			0	224	18	98		
Sc.	83	158	140	113			22	91	114	135		
Un.	88	133	153	115			0	247	337	233		
Ke.	103	138	128	112			34	163	174	67		
Hig.	100	148	88	120			14	241	42	70		
Con.	90	133	135	115			11	104	45	36		
Pop.	93	148	143	95			39	67	101	79		
Ry.	90	143	150	110			11	84	107	81		
Al.	96	296	134	44			14	188	342	42		
						Mean	21	143	139	106		
B. *Early maturity-onset diabetes*												
Ri.	245	346	436	472			51	123	173	179		
Wa. D.	96	178	218	150			6	32	97	166		
Mor.	93	110	180	218			3	77	158	300		
Moh.	95	145	193	135			59	162	339	355		
Ko.	93	240	360	120			3	14	294	364		
Fl.	138	173	248	266			56	70	190	270		
Fel.	100	152	190	141			19	107	121	102		
Fr.	113	238	310	195			35	113	316	378		
Sh.	118	232	300	190			13	38	175	76		
Go.	100	146	218	173			22	54	156	216		
Bl.	114	168	236	223			19	59	108	221		
Ma.	163	240	256	320			51	46	35	54		
We.	96	178	218	150			6	32	97	166		
Qu.	93	155	180	178			56	120	121	283		
Ok.	143	202	244	204			11	24	79	160		
Ha.	110	155	193	200			50	154	185	482		
Cr.	92	200	245	225			0	31	75	70		
Mi.	105	180	211	170			25	117	112	187		
Ro.	113	177	233	240			0	182	224	266		
Wo.	100	163	170	180			42	160	168	207		
Poi.	103	188	243	190			5	17	83	173		
Fo. F.	152	244	266	380			11	22	28	23		
No.	105	195	215	185	91	83	59	190	238	392	159	59
Ny.	93	158	193	138	60		11	53	90	154	17	
Moo.	113	153	205	268			63	148	91	131		
Fo. J.	90	169	193	185	83		34	238	249	490	173	
Br.	90	167	198	163	86	67	20	62	133	201	79	29
Pl.	100	183	193	160	63		42	168	241	308	191	
Mu.	130	233	326	374			3	17	68	126		
Fla.	93	140	170	178			22	140	126	220		

TABLE III—*Continued*

Subj.	Blood sugars (mg/100 ml) F	0.5 hr	1 hr	2 hrs	3 hrs	4 hrs	Plasma insulin concentrations (μU/ml) F	0.5 hr	1 hr	2 hrs	3 hrs	4 hrs
B. *Early maturity-onset diabetes*—Continued												
Car.	108	165	238	183	90		42	34	106	247	106	
How.	125	198	223	250			14	132	140	245		
Coo.	95	145	163	178	128		28	213	205	350	233	
Fele.	100	193	266	235			0	31	84	81		
Le.	90	173	250	193			65	65	294	392		
Ga.	105	148	178	135			70	148	210	302		
Hor.	95	165	147	195	211		8	148	140	364	386	
Eh.	91	156	211	309	246		17	129	176	545	531	
						Mean	27	97	156	243		
C. *Undetermined status*												
Wa. D.	85	100	150	148			0	35	56	42		
Wr.	98	123	158	138			27	86	75	75		
Ric.	100	138	158	143			13	140	226	100		
Ba.	88	140	150	129			14	144	230	405		
Har.	88	148	178	140			36	187	232	134		
Hew.	83	145	163	105			4	56	64	94		
Jos.	95	147	171	136			49	130	192	157		
Leh.	100	152	162	145			14	186	224	395		
Wis.	103	150	158	128			0	155	182	148		
Doh.	70	110	155	128			8	48	28	33		
Lut.	100	118	160	130			28	51	112	132		
Sa.	100	170	143	110			11	48	140	42		
Wi.	90	123	164	118			14	56	104	233		
						Mean	17	101	128	153		
D. *Decompensated cirrhosis*												
Man.	96	164	146	136			54	240	356	486		
Mar.	92	168	150	100			8	140	226	143		
Cara.	95	143	140	93			14	40	57	30		
Cro.	85	143	118	85			2	16	14	8		
Di.	78	100	82	65			25	33	19	5		
Fo.	80	135	100	80			5	36	22	8		
E. *Pituitary tumors*												
Sil.*	92	122	147	134			32	350	570	175		
Sin.†	90	135	140	117			5	177	192	180		
Led. (Acromegaly)	93	163	174	103			8	156	203	109		
F. *Thyrotoxicosis*												
El.	103	215	240	210			38	275	230	240		
Yo.	93	170	184	103			65	247	324	81		
G. *Others*												
Ku. (Hemochromatosis)	73	135	175	160			48	121	321	330		
Cra. (Acute pancreatitis)	100	120	138	125			5	20	100	135		
Bl.‡	48	108	148	135			28	90	56	67		
Coh.§	36	52	78	76			118	190	199	98		

* Chromophobe adenoma.
† Eosinophilic and chromophobe adenoma with acromegaly.
‡ Hypoglycemia, cause undetermined, after partial pancreatectomy.
§ Proven islet cell adenoma (courtesy of Dr. H. Epstein).

higher in the diabetic (mean, 27 μU per ml) than in the nondiabetic (mean, 21 μU per ml) subjects, although 34 per cent of the diabetics exceeded 40 μU per ml in contrast to only 10 per cent of the nondiabetics. In none of the 68 patients in both of these groups did the fasting level exceed 70 μU per ml (Table III, A and B, Figure 6). These values are in good agreement with those reported

earlier in a smaller series of subjects (15). The responses to orally administered glucose in diabetic and nondiabetic patients differed more markedly than did the fasting insulin concentrations. Nondiabetic subjects were about equally divided in showing the peak insulin concentration at 0.5 hour or 1 hour (Table IIIA), whereas with few definite exceptions diabetic patients showed the maximal insulin concentration at 2 hours (Table IIIB). The average insulin concentration at 0.5 hour was lower in diabetic (mean, 97 μU per ml) than in nondiabetic (mean, 143 μU per ml) subjects, but the diabetics appeared to form two groups at this point (Figure 6). A delayed insulin response is suggestive in the lower of these two groups.

Although there is a large scatter of individual values, the mean curves for the two groups illustrate these differences clearly (Figure 6). The average integrated insulin concentration during the 2 hour glucose tolerance test was 26 per cent higher for the diabetic (147 μU per ml) than for the nondiabetic (117 μU per ml) group. The "undetermined" group (Table IIIC) probably represents a mixture of early diabetic and nondiabetic subjects and nothing can be concluded definitely about the variable insulin response to glucose loading.

Insulin concentrations in four diabetic and five nondiabetic subjects given an additional 50 g of glucose at half hour intervals from 1.5 to 2.5 hours are shown in Figure 7 and Table IV. Insulin concentrations rose to higher levels in both groups but more marked increases were observed in the diabetic subjects.

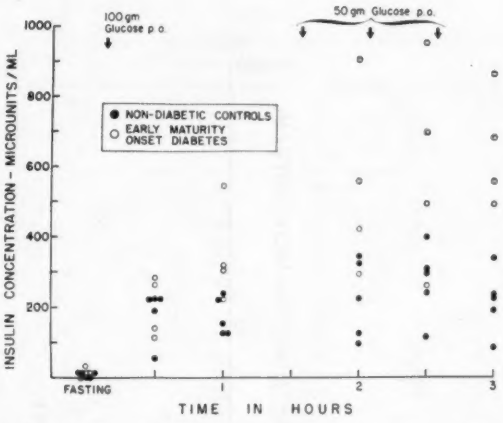

FIG. 7. PLASMA INSULIN CONCENTRATIONS DURING HEAVY GLUCOSE LOADING EXPERIMENTS IN DIABETIC AND NONDIABETIC SUBJECTS.

It should be emphasized that insulin-I^{131} when administered intravenously exhibits a rapid fall in concentration due to a marked and continuous increase in its apparent volume of distribution for a period of about 30 to 60 minutes and to a metabolic turnover rate with a half-time of about 35 minutes (22). It may be reasonably expected that endogenously secreted insulin behaves similarly,[5] and therefore that any particular peak concentra-

[5] Endogenously secreted insulin is, in addition, subject to removal by the liver before it reaches the peripheral circulation (28, 29).

TABLE IV

Effect of heavy glucose loading on blood sugars and plasma insulin levels*

Subj.	Blood sugars						Plasma insulin concentrations					
	F	0.5 hr	1 hr	2 hrs	2.5 hrs	3 hrs	F	0.5 hr	1 hr	2 hrs	2.5 hrs	3 hrs
	mg/100 ml						*μU/ml*					
Nondiabetic controls												
Hea.	84	136	106	130	108	110	3	190	129	345	308	190
Gas.	78	118	86	108	104	98	14	224	129	322	300	341
Rei.	90	127	129	105	103	101	15	224	224	125	400	225
Keh.	94	158	142	98	90	86	0	224	238	224	241	235
McC.	100	144	150	104	102	90	17	56	151	98	118	84
Maturity-onset diabetes												
Cri.	100	188	214	208	188	162	17	265	548	910	960	685
All.	86	140	152	166	126	122	12	284	223	560	496	496
Ab.	90	143	170	167	177	155	31	112	309	420	700	870
Ti.	86	170	181	155	149	145	3	140	313	294	255	578

* Glucose 100 g p.o., immediately after fasting specimen; glucose, 50 g p. o., at 1.5, 2 and 2.5 hours.

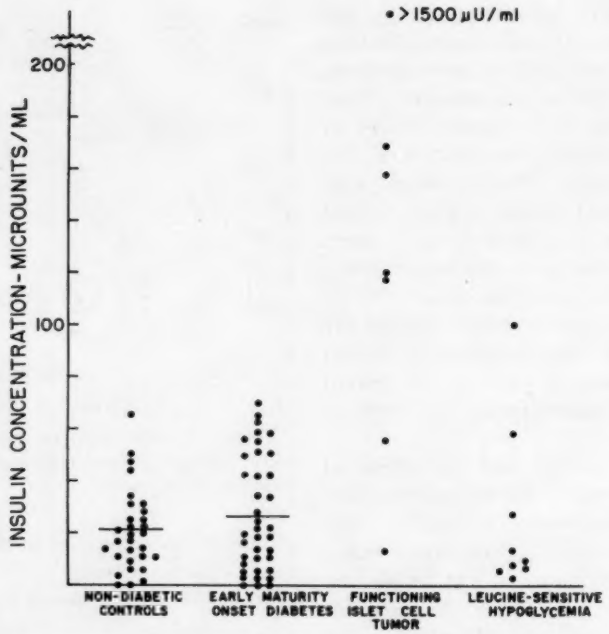

FIG. 8. FASTING PLASMA INSULIN CONCENTRATIONS IN VARIOUS GROUPS OF SUBJECTS. The subject with plasma insulin concentration greater than 1,500 μU per ml had an islet cell adenocarcinoma with widespread metastases (patient of Dr. J. Field).

tion depends on the precise moment of sampling. A very rapid and pronounced fall from the peak concentration would be anticipated in the case of a single secretory spurt. Conversely, a sustained elevation or continued rise in insulin concentration implies a continued secretion during the time interval under observation.

Insulin concentrations in patients with islet cell tumors or leucine-induced hypoglycemia. Insulin concentrations in fasting plasmas from five of seven patients [6] with proven islet cell tumors were elevated above normal levels (Figure 8), but the response to glucose was normal in the one patient studied during a glucose tolerance test (Coh., Table IIIG).

Four of six subjects [7] with leucine-induced hypoglycemia showed increased insulin concentrations following administration of L-leucine (75 to 150 mg per kg) in six of nine experiments (Figure 9), although fasting insulin concentrations were elevated in only a single patient (Figure 8), the only adult in the series and the one patient suspected on clinical grounds to have an islet cell tumor.[8] The peaks of insulin concentration, when observed, were in good time correspondence with the induced hypoglycemia.

Plasma insulin in cirrhosis, acromegaly and hyperthyroidism. Six patients with decompensated cirrhosis were studied (Table IIID). In two cases the glucose curves were high, but not within the diabetic range, and were associated with relatively high insulin concentrations. In three cases insulin concentrations were very low throughout the 2 hour glucose tolerance test, and in two of these the glucose concentration curves were quite flat. Intravenous glucose tolerance tests are necessary before it can be decided whether the observed association in the latter cases is to be at-

[6] We are indebted to Doctors H. Epstein, J. Field, E. D. Furth, E. Gordon, A. Renold and J. Steinke for these sera.

[7] We are indebted to Doctors A. DiGeorge, M. Goldner, M. Grumbach, I. Rosenthal and S. Weisenfeld for these sera.

[8] Courtesy of Doctors S. Weisenfeld and M. Goldner.

tributed to poor glucose absorption or to heightened insulin sensitivity.

In three patients with pituitary tumors, two of whom had clinical acromegaly, and in two thyrotoxic subjects, insulin concentrations during the glucose tolerance test were in the high normal range (Table III, E and F).

Results in a few individual cases that do not fall into the other categories are also included in Table IIIG.

In the absence of glucose loading, plasma insulin concentrations did not change significantly in two control subjects (Fra. and Gre., Table V).

In seven cases, sera were refrozen and repeat determinations were performed one or more months later with a different lot of insulin-I^{131}. The reproducibility of determinations performed under these conditions is shown in Figure 10.

TABLE V

*Blood sugar and plasma insulin concentrations in the absence of glucose loading**

Nondiabetic controls	Time	Blood sugar	Plasma insulin concentrations
	min	*mg/100 ml*	*μU/ml*
Gre.	0	93	17
	20	90	19
	40	90	18
	60	88	18
	120	78	25
Fra.	0	85	3
	20	85	5
	40	80	5
	60	85	3
	120	88	3

* Subjects were fasted overnight and throughout the period of blood sampling.

DISCUSSION

The demonstration that unlabeled insulin could displace insulin-I^{131} from complexes with insulin-binding antibody (*22*) and that the fraction of insulin-I^{131} bound to antibody decreases progressively with increase in insulin concentration (*22*) laid the foundation for the immunoassay of insulin employing isotopically labeled insulin. In initial reports describing results with the present method for immunoassay of beef insulin (*9, 10*) it was emphasized that species differences in the reaction of insulin with insulin antisera exist and that

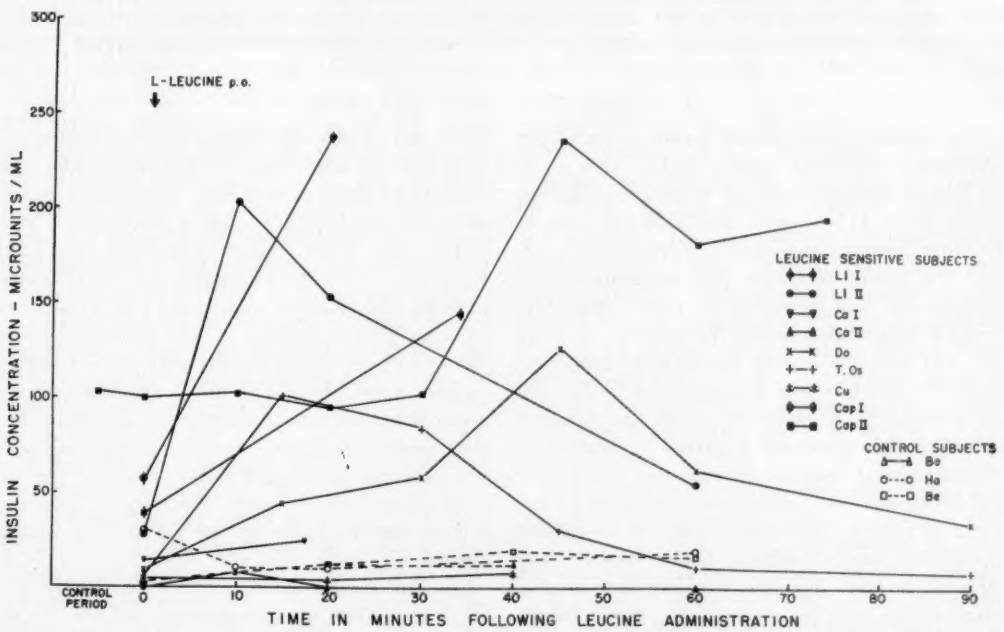

FIG. 9. PLASMA INSULIN CONCENTRATIONS FOLLOWING ADMINISTRATION OF L-LEUCINE TO CONTROL AND LEUCINE-SENSITIVE HYPOGLYCEMIC SUBJECTS.

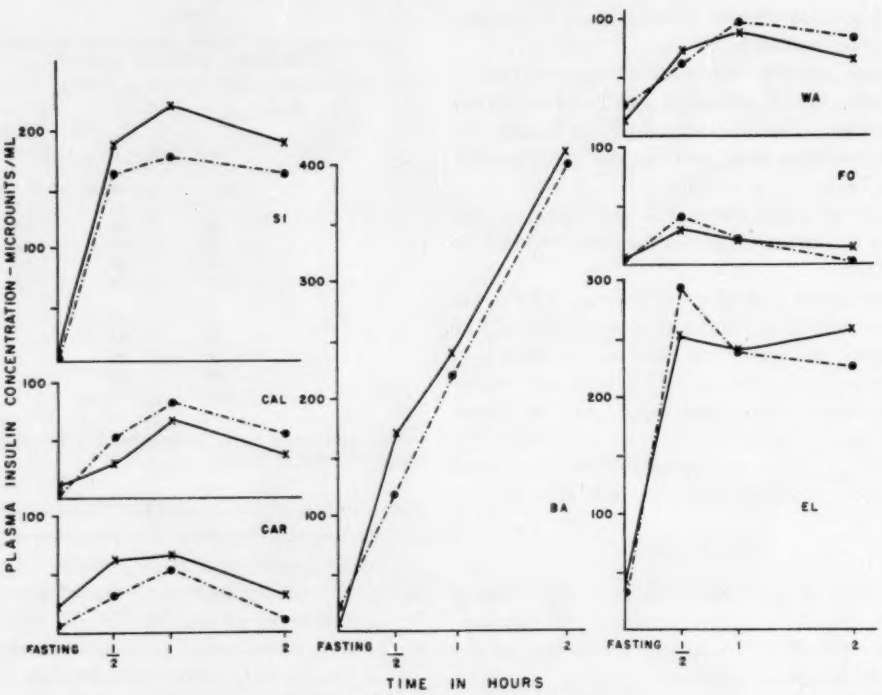

FIG. 10. REPRODUCIBILITY OF PLASMA INSULIN DETERMINATIONS ON THE SAME PLASMA SAMPLES PERFORMED ONE OR MORE MONTHS APART WITH DIFFERENT LOTS OF INSULIN-I^{131}. Plasmas were stored frozen between determinations. In these cases, the average values of the two determinations are presented in Table III.

human antibeef, pork insulin serum is useful for microassay of animal insulins (10). However, the human antisera react too weakly with human insulin (7, 8, 11) to serve as a basis for assay of the latter hormone in plasma. Fortunately, however, the serum of guinea pigs immunized with beef insulin was reported to react sufficiently strongly with human insulin for purposes of assay (11, 14) and this finding has recently been confirmed by Grodsky and Forsham (30). The latter workers have employed a salt fractionation technique that produces a partial separation of antibody-bound insulin-I^{131} from free insulin-I^{131} and have measured insulin concentrations in extracts of plasma. However, since, in the assays of Grodsky and Forsham, human insulin was assumed to react quantitatively like beef insulin, which is not valid for guinea pig antibeef insulin serum, their absolute values for human plasma insulin concentrations are questionable. Furthermore, since the *relative* degree to which human insulin and beef insulin react at different insulin concentrations varies several-fold, even relative values obtained with beef insulin as a standard are subject to large errors. Grodsky and Forsham (30) were unable to detect insulin in most fasting plasmas and reported mean values of 31 μU per ml after glucose loading in five patients. These values are very much smaller than those reported earlier by us (14) or those of the much larger series presented here. However, a significant underestimate of human insulin concentration is precisely the anticipated consequence of the invalid assumption of equal reactivity of beef and human insulins in the guinea pig antibeef insulin system.

To our knowledge there have been only two other immunologic methods employed for the assay of insulin. Arquilla and Stavitsky (31) developed an assay for insulin based on the inhibi-

tion of hemolysis of insulin-sensitized red blood cells; however, the lower limit of detectability by this technique was approximately 0.1 μg (2.8 mU) making it unsuitable for determination of plasma insulin. Loveless (32) has used certain normal human subjects, in whom the skin can be locally sensitized to insulin (by the intracutaneous injection of human anti-insulin serum) to assay insulin by the whealing response obtained. Aside from the inconvenience associated with this method, the lower limit of detectability was 200 μU beef insulin per ml and human plasma insulin was not detectable, a result attributed in part to the lesser reactivity of human insulin (32).

Reported estimates of plasma insulin concentrations, derived from the various biological assay procedures, have varied widely. Thus, the *in vitro* diaphragm assay has yielded values ranging from 40 to 80 μU per ml (33) to as high as 4,600 μU per ml (34) in fasting plasmas and from about 130 to 800 μU per ml (33) to 9,000 to 22,000 μU per ml (35) after glucose in normal subjects. Measuring the increase in oxidation of glucose-1-C^{14} to $C^{14}O_2$ by rat epididymal adipose tissue *in vitro,* Martin, Renold and Dagenais (3) found that the insulin-like activity of fasting normal plasma in this preparation corresponded to 50 to 350 μU of insulin per ml. Pfeiffer, Pfeiffer, Ditschuneit and Ahn (36), using the same assay, found that plasma diluted 1:2 gave higher and more consistent insulin concentrations and reported normal fasting levels of 135 to 680 μU per ml in 15 normal human subjects, with concentrations frequently exceeding 2,000 to 4,000 μU per ml after tolbutamide and metahexamide. Employing the immunoassay method we have observed generally much smaller increases in peripheral insulin concentration after large doses of sodium tolbutamide, administered intravenously or by mouth, than after glucose given by the same routes to normal or diabetic men (37).

It is generally agreed (5, 34) that dilution of plasma or serum increases markedly the estimated insulin concentration in the diaphragm assay and similar observations have been made in the rat epididymal fat pad assay (36). This phenomenon has been either attributed to the presence of inhibitory substances in the plasma (5, 34) or interpreted as indicating that insulin-like activity of serum as measured by the isolated rat diaphragm is not specific for insulin per se. Randle has found that albumin and other proteins may exert a nonspecific stimulation of glucose uptake by rat diaphragm (5) and that 1 ml of plasma exhibiting an insulin concentration of 13,000 μU per ml in the diaphragm assay had no effect on blood sugar when injected into alloxan-diabetic hypophysectomized rats, whereas 2,000 μU insulin produced a marked hypoglycemia (35).

The recent report by Leonards (6), that insulin-like activity in plasma, when tested on the rat epididymal fat pad, persists after total pancreatectomy and that insulin-neutralizing antiserum from guinea pigs has no inhibitory effect on the insulin-like activity of human serum in this system, has raised a serious question as to what part of the insulin-like effect on fat tissue is due to insulin itself.

In vivo insulin assays have also yielded variable estimates of plasma insulin concentration. Measuring the fall in blood sugar induced in adrenodemedullated alloxan-diabetic hypophysectomized rats, Anderson, Lindner and Sutton (38) were unable to detect circulating insulin in fasting plasma although the method was sensitive to 125 μU insulin. Bornstein and Lawrence (1) using adrenalectomized hypophysectomized diabetic rats reported plasma insulin concentrations 2 hours after glucose to average about 340 μU per ml in normal subjects and 100 to 320 μU per ml in diabetic patients not subject to ketosis, but Randle (5) was unable to confirm the suitability of these animals for insulin assay. More recently, Baird and Bornstein (39), employing adrenalectomized alloxan-diabetic mice, have found that normal fasting plasma extracted with acid ethanol-*n*-butanol-toluene (which is thought to separate insulin from insulin antagonists) contains about 1,000 μU per ml. Values about three times as high were observed after glucose feeding. Values as high or higher were found in four of six diabetic subjects.

At the present time it does not appear possible to resolve all the apparently divergent findings summarized here. It is necessary, however, to point out that plasma insulin concentrations determined by the immunoassay technique are in agreement with the lowest estimates derived from other methods of assay, notably the *in vivo* bioassays of Bornstein and Lawrence (1), and of Anderson

and co-workers (38), and the diaphragm assay of Vallance-Owen and Hurlock (33). By comparison with the biological effects of exogenous insulin the lower concentrations appear most reasonable.

A rough estimate of the amount of insulin secreted can be derived from the insulin concentrations reported here according to the following considerations. From the area under the mean insulin curve in nondiabetic subjects it is found that the average insulin concentration during the 2 hour period following glucose administration was 117 μU per ml. It has been shown previously (22) that I^{131}-labeled insulin in man is metabolized at a rate of about 2 per cent per minute and is distributed into an apparent volume of distribution of about 37 per cent of body weight in about 45 minutes, distribution being about half completed at 15 minutes. If we now assume that the distribution and metabolism of endogenous insulin that reaches the peripheral circulation is similar quantitatively to that of exogenous insulin [9] and make the conservative estimate that, on the average, the endogenous insulin was distributed in a volume corresponding to 30 per cent of body weight (21 L) over the 2 hour period, we can calculate that 0.117 U per L $\times$ 21 L was being degraded at the rate of 2 per cent per minute during these 120 minutes. This computation leads to the estimate that approximately 6 U of insulin reached the peripheral circulation during the 2 hour glucose tolerance test. Madison, Combes, Unger and Kaplan (28) have found that approximately 50 per cent of insulin given into the portal vein is removed from the circulation during its first passage through the liver, and this value is in good agreement with the figure of 40 per cent given by Mortimore and Tietze in the rat (29). If we accept the 50 per cent value for the liver of man, it can be concluded that an average of about 12 U of insulin was secreted during the glucose tolerance test in nondiabetic subjects. This is what might be expected in each of three feedings per day. If, also, there is added into the calculation (on the same basis) the amount of insulin necessary to maintain a fasting level of 0.021 U per L for the other 18 hours, we arrive at the estimate of 36 U (postprandial secretion) plus 19 U (fasting secretion) = 55 U for the average total insulin secretion per day in nondiabetic subjects.[10] Since, even at the end of the 2 hour glucose tolerance test the insulin concentration was still above fasting levels, calculations over a more extended time period would yield even slightly higher estimates. These figures are certainly consistent with the insulin requirement of 30 to 40 U daily in totally depancreatized human subjects (40), since exogenously administered insulin does not experience the initial hepatic removal to which endogenously secreted insulin is subjected.

In order to resolve the much higher estimates of plasma insulin concentration given by Willebrands, van der Geld and Groen (34), Randle (35), and Pfeiffer and associates (36) with these considerations, we must assume either that the turnover rate of endogenous insulin is very much slower than that of exogenously administered insulin (in which event it is difficult to understand why human subjects do not remain in prolonged jeopardy of hypoglycemia from the high insulin concentrations that follow glucose administration) or that endogenous insulin is confined almost exclusively to the plasma. Even if the latter alternative (which also is in strong conflict with the results on distribution of exogenous insulin) were true, a fasting level as high as 4,600 μU per ml (34) would mean that there is almost 14 U of insulin in the circulation of fasting human subjects, a conclusion which is still difficult to accept. Randle's (35) values of 9,000 to 22,000 μU per ml in normal plasma 2.5 hours following glucose would mean a total of 27 to 66 U in plasma alone, neglecting insulin in extravascular space, at a time when the blood sugar is usually at a normal level. However, as already noted, Randle has indicated his conviction that this "insulin-like" activity is not due entirely to insulin alone (5).

To return now to the results of the present study, it will be noted that the high insulin con-

[9] It has been established that exogenous unlabeled crystalline beef insulin and I^{131}-labeled crystalline beef insulin show virtually identical plasma disappearance curves in the rabbit (10). Furthermore, the precipitous fall in insulin concentration from peak levels, observed in many patients of the present study (Table IIIA), even when insulin secretion may be presumed to be continuing, is evidence that endogenous insulin also is rapidly removed from the circulation.

[10] These calculations ignore any increase in insulin secretion that would result from small feedings between meals.

centrations observed in diabetic subjects during the glucose tolerance test are not inconsistent with the less extensive data of Bornstein and Lawrence (1) and Baird and Bornstein (39). Very recently Seltzer and Smith (41), employing the rat diaphragm assay of Vallance-Owen and Hurlock (33), have reported insulin concentrations one hour after glucose, in tolbutamide-sensitive adult diabetics, almost in the normal range, but significantly lower values were observed in juvenile diabetics and adult tolbutamide-insensitive diabetics. To resolve the present finding of a higher than normal integrated insulin output in diabetics during the glucose tolerance test with sustained hyperglycemia in these patients, it must be concluded that the tissues of the maturity-onset diabetic do not respond to his insulin as well as the tissues of the nondiabetic subject respond to his insulin. However, from these observations it cannot be concluded that the early diabetic has the same maximal potential insulin output as the nondiabetic, since in the latter the return of blood sugar to normal levels does not allow for the continued stimulus of prolonged hyperglycemia as in the diabetic. The attempt to produce a sustained stimulus to insulin secretion by repeated administration of glucose to a total of 250 g did result in a more marked insulin secretion in nondiabetic subjects. However, the response of diabetics was still greater indicating that their insulin reserve is not depleted during the 100 g glucose tolerance test. The experiments failed, however, to test maximal insulin secretory capacity of the nondiabetic subjects since a sustained hyperglycemia was not achieved in these patients.

Appreciation of the lack of responsiveness of blood sugar, in the face of apparently adequate amounts of insulin secreted by early maturity-onset diabetic subjects, is obviously of importance in the interpretation of the pathogenesis of this type of diabetes. However, the data at hand can only indicate that absolute insulin deficiency per se is not the cause of the hyperglycemia and suggest other possibilities that merit investigation, namely, *1*) abnormal tissues with a high threshold for the action of insulin; *2*) an abnormal insulin that acts poorly with respect to hormonal activity *in vivo* but reacts well immunologically *in vitro; 3*) an abnormally rapid inactivation of hormonally active sites [a suggestion in accord with the ideas expressed by Mirsky (42)] but not of immunologically active sites on the insulin molecules; and *4*) the presence of insulin antagonists. The last suggestion has been made many times by previous workers. A joint attack on the problem, utilizing both the specific immunoassay for plasma insulin and an assay method that measures the net biological effect of insulin and its inhibitors would seem to be indicated.

The high fasting insulin concentrations observed in hypoglycemia associated with functioning islet cell tumors are not unexpected. However, the normal response to glucose in the one patient studied suggests that the insulin-producing tumor may be secreting insulin continuously or sporadically but that it is not stimulated specifically by hyperglycemia. The failure to detect high plasma concentrations of insulin in two cases can possibly be explained by the normally rapid turnover of insulin and the sampling at a time when insulin production by the tumor had been quiescent for an hour or two previously.

Leucine-induced hypoglycemia in children with idiopathic hypoglycemia was first reported by Cochrane, Payne, Simpkiss and Woolf (43) but a satisfactory interpretation of the disturbance has not been given. From the results of the present study it appears that leucine serves as an abnormal stimulus to insulin secretion in these subjects but may also have other effects. Most of the patients whose sera were assayed here have been studied in detail in other respects as well by the various investigators who supplied the sera and are to be reported by them individually.

SUMMARY AND CONCLUSION

1. An immunoassay for plasma insulin in man is presented, based on the reaction of human insulin, competing with beef insulin-I^{131}, with insulin-binding antibodies in the sera of guinea pigs immunized with beef insulin. The method is sensitive to less than 1 μU of insulin, permitting measurement of insulin concentrations in 10 to 20 μl of plasma.

2. Human insulin added *in vitro* to plasma is recovered quantitatively, and measured endogenous insulin concentrations decrease proportionately on dilution of plasma over the range 1:2 to 1:100.

3. Endogenous plasma insulin is destroyed by incubation with cysteine and endogenous insulin adsorption by cellulose columns is quantitatively similar to the adsorption of added beef insulin-I^{131}.

4. Repeat determinations of insulin concentrations on the same plasma samples (stored frozen in the interim) one or more months apart, with different lots of insulin-I^{131}, were generally in good agreement.

5. Fasting plasma insulin concentrations in early maturity-onset diabetic patients who had never been treated with insulin, (mean, 27 μU per ml) and in nondiabetic subjects (mean, 21 μU per ml) did not differ markedly. Following 100 g of glucose by mouth, nondiabetic subjects usually showed peak insulin concentrations at 0.5 hour (mean, 143 μU per ml) or 1 hour (mean, 139 μU per ml) and a decline by 2 hours (mean, 106 μU per ml). In contrast, insulin concentrations in diabetic subjects showed a lesser increase at 0.5 hour (mean, 97 μU per ml) but continued to rise to a peak at 2 hours (mean, 243 μU per ml). The integrated average insulin concentration during the 2 hour glucose tolerance test was 26 per cent higher in diabetics (mean, 147 μU per ml) than in nondiabetics (mean, 117 μU per ml).

6. In a small series of patients subjected to additional glucose loading at 1.5, 2 and 2.5 hours, very high insulin concentrations were observed in both groups, but levels in diabetic patients far exceeded those in nondiabetic subjects.

7. Fasting insulin concentrations were elevated in five of seven subjects with functioning islet cell adenomas but insulin secretory response to glucose was normal in the one patient studied.

8. Four of six subjects with leucine-sensitive hypoglycemia showed increased insulin concentrations following administration of leucine in six of nine experiments.

9. Insulin responses were generally in the high normal range in three patients with pituitary tumors (two associated with acromegaly) and in two patients with thyrotoxicosis.

10. Plasma insulin concentrations measured by imunoassay are compared with values obtained by other assay methods and found to yield the lowest estimates.

11. Calculation of the average normal daily insulin secretion rate, on the basis of the data presented, yields an estimate of about 55 U of insulin per day.

ACKNOWLEDGMENTS

We are indebted to the investigators mentioned, who sent us sera on the unusual cases reported here. We also wish to thank Mr. Manuel Villazon for technical assistance, Mr. Paul Newman for the charts, Mr. David Lubin, Mr. Glenn Harahan and Mr. Lawrence Steur for the figures, and Miss Eve Spelke and Mrs. Frieda Steiner for secretarial assistance. Finally, the cooperation of the Medical and Laboratory Services of the Bronx Veterans Administration Hospital is gratefully acknowledged.

REFERENCES

1. Bornstein, J., and Lawrence, R. D. Plasma insulin in human diabetes mellitus. Brit. med. J. 1951, **2**, 1541.
2. Groen, J., Kamminga, C. E., Willebrands, A. F., and Blickman, J. R. Evidence for the presence of insulin in blood serum. A method for an approximate determination of the insulin content of blood. J. clin. Invest. 1952, **31**, 97.
3. Martin, D. B., Renold, A. E., and Dagenais, Y. M. An assay for insulin-like activity using rat adipose tissue. Lancet 1958, **2**, 76.
4. Baird, C. W., and Bornstein, J. Plasma-insulin and insulin resistance. Lancet 1957, **1**, 1111.
5. Randle, P. J. Insulin in blood. Ciba Found. Coll. Endocr. 1957, vol. XI, p. 115.
6. Leonards, J. R. Insulin-like activity of blood. What it is. Fed. Proc. 1959, **18**, 272.
7. Berson, S. A., and Yalow, R. S. Cross reactions of human anti-beef pork insulin with beef, pork, sheep, horse and human insulins. Fed. Proc. 1959, **18**, 11.
8. Berson, S. A., and Yalow, R. S. Species-specificity of human anti-beef, pork insulin serum. J. clin. Invest. 1959, **38**, 2017.
9. Berson, S. A. *In* Résumé of Conference on Insulin Activity in Blood and Tissue Fluids, R. Levine and E. Anderson, Eds. Bethesda, Md. 1957, p. 7.
10. Berson, S. A., and Yalow, R. S. Isotopic tracers in the study of diabetes *in* Advances in Biological and Medical Physics, J. H. Lawrence and C. A. Tobias, Eds. New York, Academic Press Inc., 1958, vol. VI, p. 349.
11. Berson, S. A., and Yalow, R. S. Recent studies on insulin-binding antibodies. Ann. N. Y. Acad. Sci. 1959, **82**, 338.
12. Berson, S. A., and Yalow, R. S. Immunoassay of insulin *in* Hormones in Human Plasma, H. N. Antoniades, Ed. Boston, Little, Brown & Co. In press.
13. Berson, S. A., and Yallow, R. S. Immunologic reactions to insulin *in* Diabetes, R. H. Williams, Ed. New York, Paul B. Hoeber, Inc. 1960, p. 272.

14. Yalow, R. S., and Berson, S. A. Assay of plasma insulin in human subjects by immunological methods. Nature (Lond.) 1959, **184**, 1648.
15. Yalow, R. S., and Berson, S. A. Plasma insulin concentrations in non-diabetic and early diabetic subjects determined by a new sensitive immunoassay technique. Diabetes. In press.
16. Bauman, A., Rothschild, M. A., Yalow, R. S., and Berson, S. A. Distribution and metabolism of I^{131} labeled human serum albumin in congestive heart failure with and without proteinuria. J. clin. Invest. 1955, **34**, 1359.
17. Pressman, D., and Eisen, H. N. The zone of localization of antibodies. V. An attempt to saturate antibody-binding sites in mouse kidney. J. Immunol. 1950, **64**, 273.
18. Yalow, R. S., and Berson, S. A. Effect of x-rays on trace-labeled I^{131}-insulin and its relevance to biologic studies with I^{131}-labeled proteins. Radiology 1956, **66**, 106.
19. Berson, S. A., and Yalow, R. S. Radiochemical and radiobiological alterations of I^{131}-labeled proteins in solution. Ann. N. Y. Acad. Sci. 1957, **70**, 56.
20. Ferrebee, J. W., Johnson, B. B., Mithoefer, J. C., and Gardella, J. W. Insulin and adrenocorticotropin labeled with radio-iodine. Endocrinology 1951, **48**, 277.
21. Newerly, K., and Berson, S. A. Lack of specificity of insulin-I^{131} binding by isolated rat diaphragm. Proc. Soc. exp. Biol. (N. Y.) 1957, **94**, 751.
22. Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K. Insulin-I^{131} metabolism in human subjects: Demonstration of insulin binding globulin in the circulation of insulin-treated subjects. J. clin. Invest. 1956, **35**, 170.
23. Berson, S. A., and Yalow, R. S. Quantitative aspects of the reaction between insulin and insulin-binding antibody: Relation to problem of insulin resistance. J. clin. Invest. 1959, **38**, 1996.
24. Field, J. B., Tietze, F., and Stetten, D., Jr. Further characterization of an insulin antagonist in the serum of patients in diabetic acidosis. J. clin. Invest. 1957, **36**, 1588.
25. Fisher, A. M. Personal communication.
26. Fajans, S. S., and Conn, J. W. The early recognition of diabetes mellitus. Ann. N. Y. Acad. Sci. 1959, **82**, 208.
27. Somogyi, M. Determination of blood sugar. J. biol. Chem. 1945, **160**, 69.
28. Madison, L. L., Combes, B., Unger, R. H., and Kaplan, N. The relationship between the mechanism of action of the sulfonylureas and the secretion of insulin into the portal circulation. Ann. N. Y. Acad. Sci. 1959, **74**, 548.
29. Mortimore, G. E., and Tietze, F. Studies on the mechanism of capture and degradation of insulin-I^{131} by the cyclically perfused rat liver. Ann. N. Y. Acad. Sci. 1959, **82**, 329.
30. Grodsky, G., and Forsham, P. An immunochemical assay of total extractable insulin in man. J. clin. Invest. 1960, **39**, 000.
31. Arquilla, E. R., and Stavitsky, A. B. The production and identification of antibodies to insulin and their use in assaying insulin. J. clin. Invest. 1956, **35**, 458.
32. Loveless, M. H. A means of estimating circulating insulin in man. Quart. Rev. Allergy 1956, **10**, 374.
33. Vallance-Owen, J., and Hurlock, B. Estimation of plasma-insulin by the rat diaphragm method. Lancet 1954, **1**, 68.
34. Willebrands, A. F., v. d. Geld, H., and Groen, J. Determination of serum insulin using the isolated rat diaphragm. The effect of serum dilution. Diabetes 1958, **7**, 119.
35. Randle, P. J. Assay of plasma insulin activity by the rat-diaphragm method. Brit. med. J. 1954, **1**, 1237.
36. Pfeiffer, E. F., Pfeiffer, M., Ditschuneit, H., and Ahn, C. Clinical and experimental studies of insulin secretion following tolbutamide and metahexamide administration. Ann. N. Y. Acad. Sci. 1959, **82**, 479.
37. Yalow, R. S., Black, H., Villazon, M., and Berson, S. A. Comparison of plasma insulin levels following administration of tolbutamide and glucose. Diabetes. In press.
38. Anderson, E., Lindner, E., and Sutton, V. A sensitive method for the assay of insulin in blood. Amer. J. Physiol. 1947, **149**, 350.
39. Baird, C. W., and Bornstein, J. Assay of insulin-like activity in the plasma of normal and diabetic human subjects. J. Endocr. 1959, **19**, 74.
40. Goldner, M. G., and Clark, D. E. The insulin requirement of man after total pancreatectomy. J. clin. Endocr. 1944, **4**, 194.
41. Seltzer, H. S., and Smith, W. L. Plasma insulin activity after glucose. An index of insulogenic reserve in normal and diabetic man. Diabetes 1959, **8**, 417.
42. Mirsky, I. A. The etiology of diabetes mellitus in man. Recent Progr. Hormone Res. 1952, **7**, 437.
43. Cochrane, W. A., Payne, W. W., Simpkiss, M. J., and Woolf, L. I. Familial hypoglycemia precipitated by amino acids. J. clin. Invest. 1956, **35**, 411.

THE RELATION OF DIAPHORASE OF HUMAN ERYTHROCYTES TO INHERITANCE OF METHEMOGLOBINEMIA

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Hereditary methemoglobinemia has been found to have a relatively high prevalence in Alaskan Eskimos and Indians (1). Persons with this condition have been found to have a complete lack of diphosphopyridine nucleotide diaphorase activity in red cells (2).

Of the 21 known cases of methemoglobinemia in 13 families in Alaska, 12 cases in 7 families occurred in Ingalik Indians, a small group of about 800 Athabascans living on the Innoko, Yukon and Kuskokwim Rivers. The present study is concerned with a study of diaphorase levels of red cells of 271 of these people and of 47 Eskimos living nearby. Evidence is presented which indicates that parents of methemoglobinemic children have about half the normal level of erythrocyte diaphorase, and that the condition is inherited as a simple recessive trait.

METHODS

Determination of erythrocyte diaphorase. Five ml of blood was collected and mixed with 1 ml acid citrate dextrose (ACD) solution (1.47 g glucose, 1.32 g sodium citrate and 0.44 g citric acid per 100 ml water). Other anticoagulants can be used but we have preferred ACD since the diaphorase level could not be determined until several days after the blood was collected. In the presence of ACD, the diaphorase level of cells was unchanged over a period of at least 2 weeks. The serum was drawn off and the cells were washed 3 times with 10 volumes of phosphate-buffered saline solution (9 volumes 0.9 per cent NaCl plus 1 volume 0.1 M potassium phosphate, pH 7.3). To the cells was added an equal volume of phosphate-buffered saline solution containing 1 per cent sodium nitrite and the mixture was shaken and allowed to stand for 20 minutes. The cells were then washed 5 more times with 10 volumes of phosphate-buffered saline solution to completely remove the nitrite.

To 0.1 ml of the oxidized cells was added 1.9 ml water. After shaking, the mixture was centrifuged and the hemolysate decanted from the insoluble matter. The methemoglobin content of the hemolysate was determined by measuring absorption at 600 mμ after 1:15 dilution; it was found by comparison with the method of Evelyn and Malloy (3) that an optical density of 0.2 at 600 mμ in a 10 mm cell was given by 1.08 mg methemoglobin per ml.

To each of two cuvets were added 0.05 ml 1 M Tris (hydroxymethyl)aminomethane (pH 7.55), 0.1 ml 0.01 M sodium ethylenediamine tetraacetate (EDTA) and 0.05 ml 0.0012 M 2,6-dichlorobenzenoneindophenol (Eastman). To one cuvet was added hemolysate equivalent to 3.25 mg methemoglobin and water to a volume of 2.98 ml; to the blank was added 2.78 ml water. The reaction was started by addition of 0.02 ml 0.0088 M reduced diphosphopyridine nucleotide (DPNH). Optical density in a 10 mm cell was measured at 3-minute intervals for 21 minutes at 600 mμ in a Beckman spectrophotometer. The spectrophotometer was equipped with a water jacket, through which water at 25° C was circulated.

Under these conditions, the decrease in optical density both with hemolysate and in the blank was nearly linear. The level of diaphorase activity of the hemolysate was taken to be the rate of reaction expressed as change in absorbance per minute (ΔA per minute) corrected for the blank.

RESULTS

A typical plot of results obtained with this method is shown in Figure 1. It will be noted that the rate of reduction with dye in the presence of methemoglobinemic hemolysate is not distinguishable from the blank rate. The net rate of reduction of 2,6-dichlorobenzenoneindophenol has been determined in 14 methemoglobinemic persons representing 11 families, and 13 parents of methemoglobinemic children representing 8 families. The results are summarized in Table I, and compared with those from 88 children of normal parents. The level of diaphorase in red cells of men did not differ from that of women, and there was no dependence on age.

Determinations of diaphorase activity have been made on 271 Ingalik Indians and 47 Eskimos with the results shown in Figure 2. It is apparent from this figure that the levels fall into three groups. The first consists of clearly distinguishable levels between − 0.0001 and 0.0005 ΔA per minute and represents only methemoglobinemic persons.

The second group begins at levels of 0.0015 ΔA per minute and extends to about 0.0030 ΔA per minute; all parents of methemoglobinemic children were in this group. The third and largest group consisted of levels higher than about 0.0030 ΔA per minute.

Assuming that levels lower than 0.0010 are characteristic of the methemoglobinemic homozygote, *mm;* levels between 0.0010 and 0.0030 represent the heterozygote, *Mm;* and levels in excess of 0.0030 the normal homozygote, *MM:* the results in 45 families can be summarized as shown in Table II. A considerable proportion of the persons with low levels is related; the relationship is shown in Figure 3.

TABLE I

Net rate of dye reduction in methemoglobinemic persons; parents of methemoglobinemic children, and children of normal parents

Subjects	No.	Rate of dye reduction* Mean	SD	Range
Methemoglobinemic	14	0.9	2.1	−3 to 5
Parents of methemoglobinemic children	13	21.8	4.4	15 to 28
Children of normal parents	88	45.5	8.5	28 to 81

* Rate of dye reduction is expressed as absorbance change per minute × 10^4.

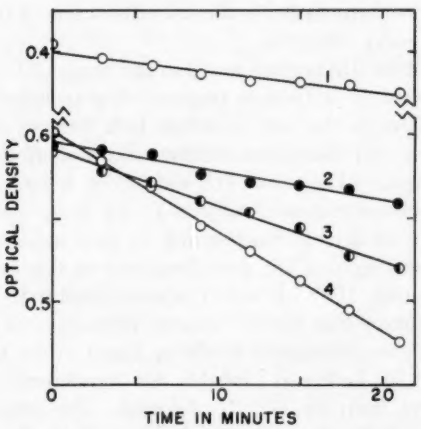

FIG. 1. RATE OF DYE REDUCTION. 1) Reaction in absence of hemolysate; 2) reaction with hemolysate from methemoglobinemic subject; 3) reaction with hemolysate from a heterozygotic sibling of 2; 4) reaction with hemolysate from a normal sibling of 2.

DISCUSSION

The method here used for determining diaphorase activity is adapted from that of Edelhoch, Hayaishi and Teply (4). In applying this method to hemolysates, it was necessary to oxidize all hemoglobin to methemoglobin, since hemoglobin itself will rapidly reduce 2,6-dichlorobenzenoneindophenol. The dye is also reduced directly by DPNH. This nonenzymatic reaction is first order with respect to dye concentration and also with respect to DPNH concentration. It is strongly dependent on pH, being very rapid at lower pH. At the pH used (7.55), the reaction is sufficiently slow so that a nearly linear reduction occurs. We have been unable to obtain a reproducible rate of nonenzymatic reduction of the dye in the absence of EDTA although this substance has no effect on the enzymatic reaction. The net rate of reaction is proportional to the amount of hemolysate added, but the amount of hemolysate that can be used is limited by the optical density of the solution.

That the factor contributed by the hemolysate is an enzyme is shown by its lability to heat and the fact that it can be purified by conventional methods of enzyme isolation. The properties of the purified enzyme will be described elsewhere. The enzyme of hemolysates is relatively stable; about one-third of the activity was lost after freezing and no further loss occurred after storage in the frozen state for six months.

The activity of the diaphorase in hemolysates is

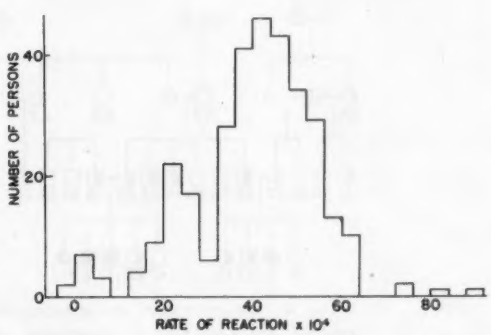

FIG. 2. NET RATE OF DYE REDUCTION IN 318 SUBJECTS. Rate of reaction is expressed as absorbance change per minute.

TABLE II

Diaphorase levels of children compared with those of parents

Parents	No. of families	No. of children with levels: <0.0010*	0.0010–0.0030*	>0.0030*
MM × *mm*	1	0	4	0
Mm × *Mm*	5	7	7	5
Mm × *MM*	10	0	7	15
MM × *MM*	29	0	1	87

* Net rate of diaphorase reaction.

strongly dependent on pH. It is more active at lower pH but the reaction cannot be conveniently determined at lower pH because the nonenzymatic reaction is also accelerated. The rate of enzymatic reaction is nearly directly proportional to initial dye concentration. Although one would anticipate that dye reduction would therefore be a first order reaction, it is nearly linear under the above conditions. The reason for this discrepancy is not known. The enzymatic reaction, unlike the nonenzymatic reaction, is nearly independent of DPNH concentration. That no diaphorase activity is present in methemoglobinemic hemolysates is shown by the fact that rate of dye reduction in the presence of these hemolysates is proportional to DPNH concentration.

It will be noted in Figure 2 that a few persons with diaphorase levels between 0.0028 and 0.0032 ΔA per minute can not unequivocally be placed in either the normal group or in the group which appears to be heterozygous. A decision on such an individual can often be reached by repeating the diaphorase assay, since the variation encountered between assays may be as high as 0.0004 ΔA per minute. (The data in Figure 2 were the results of a single determination of diaphorase activity.) Studies of the immediate family may also be helpful in reaching a decision in a doubtful case.

In spite of the fact that low levels of diaphorase may not always be readily distinguishable from normal levels, the evidence obtained from these families is clearly consistent with the view that persons who are heterozygous for methemoglobinemia have diaphorase levels about half as high as normal levels. We have not been able to detect methemoglobin in the red cells of any of these heterozygotes.

Of the 318 persons tested in this study 203 were selected by a random process—they included all families in the area in which both parents were living and there were children six or more years of age. (The other 115 were close relatives of methemoglobinemic subjects.) Of these 203, 3 were methemoglobinemic and 22 were apparently heterozygous. The gene frequency in this group was thus 0.069. It would be anticipated from this frequency that normal persons, heterozygotes and methemoglobinemics would be found in the ratio 176:26:1; this is probably not significantly different from the 178:22:3 found. The distribution of diaphorase levels shown in Table II, the data in Figure 3, and data from other families in the study are also consistent with the inheritance of methemoglobinemia as a simple recessive trait.

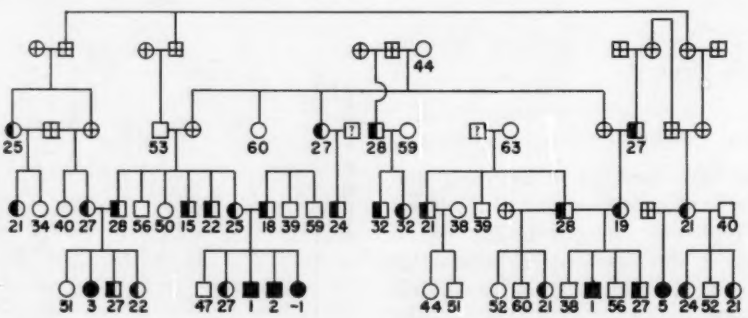

FIG. 3. RELATIONSHIP OF METHEMOGLOBINEMIC FAMILIES. The figures are net rate of dye reduction in absorbance change per minute × 10^4. Circles = females, squares = males, solid symbols = methemoglobinemic subjects, half-solid symbols = heterozygotes, open symbols = normal, crossed symbols = deceased; question mark indicates diaphorase not determined or person unknown.

SUMMARY

Diphosphopyridine nucleotide diaphorase activity is lacking in the red cells of persons with hereditary methemoglobinemia, and is present in only about half the normal amount in parents of methemoglobinemic children. Low levels of erythrocyte diaphorase appear to be characteristic of the heterozygous state of methemoglobinemia. The evidence obtained supports the conclusion that methemoglobinemia is inherited as a recessive trait.

REFERENCES

1. Scott, E. M., and Hoskins, D. D. Hereditary methemoglobinemia in Alaskan Eskimos and Indians. Blood 1958, **13**, 795.
2. Scott, E. M., and Griffith, I. V. The enzymic defect of hereditary methemoglobinemia: Diaphorase. Biochim. biophys. Acta 1959, **34**, 584.
3. Evelyn, K. A., and Malloy, H. T. Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood. J. biol. Chem. 1938, **126**, 655.
4. Edelhoch, H., Hayaishi, O., and Teply, L. J. The preparation and properties of a soluble diphosphopyridine nucleotide cytochrome *c* reductase. J. biol. Chem. 1952, **197**, 97.

ABNORMALITIES OF MUSCLE PROTEIN METABOLISM IN MICE WITH MUSCULAR DYSTROPHY *

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The discovery of a myopathic mutation in a colony of strain 129 mice (1, 2) provides an opportunity for the study of possible abnormalities in the metabolism of muscle proteins with the use of labeled amino acids. Cell proteins may show various kinds of behavior. Some of them are constantly being renewed, either secreted, like digestive enzymes or serum albumin from the liver, or subjected to a molecular turnover, as in the case of muscle aldolase and probably all the water-extractable muscle proteins. The other proteins apparently stay unchanged during the life span of the cell, as in the case of hemoglobin in red blood cells (3), of collagen in the rat (4), or of myosin in the myofibril, a subcellular unit (5, 6). We have previously shown in the rat that myosin has a very slow renewal and that its metabolic behavior permits the measurement of the life span of the myofibril, which is close to 30 days in the rat. If, after the injection of a radioactive amino acid, the radioactivity of the protein is plotted against time, the first kind of protein shows an exponential decline, but hemoglobin and myosin show a plateau followed by a rapid decline. These two kinds of curves are combined if the protein is slowly renewed and belongs to a cell or part of a cell with a relatively short life span.

In this work we have compared the metabolism of myosin and water-extractable muscle proteins in dystrophic mice, in their normal litter mates, and in another normal strain of mice. We have not prepared pure aldolase, which would have required too large a number of animals, but we have previously seen that the total water-extractable proteins have the same gross behavior as aldolase (5, 6).

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METHODS

Animals. We have used normal mice strain RAP, dystrophic mice, and their normal litter mate strain 129, which were obtained from the Roscoe B. Jackson Memorial Laboratory, where they have been isolated by Michelson, Stevens and co-workers (1, 2). The muscular dystrophy of these mice is linked to an autosomal recessive gene, the disease appearing only in the homozygotic mice. These mice have a generalized muscular atrophy, and the microscopic appearance of the muscle tissue is characteristic of progressive muscular dystrophy of man. The mice, whose weight varied between 10 and 16 g, received a diet containing 24 per cent protein. They were fasted 24 hours before and 8 hours after the injection of the radioactive amino acid. They were injected intraperitoneally with glycine-2-C^{14}, 1 μc per 10 g of body weight. For each protein preparation 3 to 4 normal mice or 4 to 5 dystrophic mice were pooled. The number of experiments was limited by the difficulty in obtaining the mice and by their short life expectancy. None of the mice lost weight during the experiments.

Preparation of the proteins. The mice were killed by bleeding. Muscles were ground and the water-extractable proteins and free glycine were extracted with twice-distilled water. The myosin was extracted with potassium iodide according to Szent-Györgyi (7) and purified twice by precipitation by dilution, and by redissolution in a buffered potassium chloride solution. The purity was checked by moving boundary electrophoresis which showed one symmetrical peak (Figure 1). The absence of actomyosin was checked in each experiment by the measurement of the viscosity before and after the addition of adenosine triphosphate. The water-extractable proteins were precipitated by adding half a volume of 20 per cent trichloroacetic acid to the muscle extract.

Free glycine of muscle. The free glycine of muscle was obtained from the supernatant of the trichloroacetic acid precipitation of the water extract. This solution was evaporated to dryness *in vacuo* and the trichloroacetic acid was extracted with ether. After treatment with dinitrofluorobenzene, the dinitrophenylglycine (DNP-glycine) was isolated on a celite column, according to

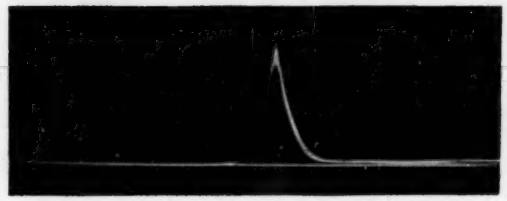

FIG. 1. ELECTROPHORESIS OF MOUSE MYOSIN. 1.19 v per cm; 22 hours, 40 minutes. Phosphate buffer $\mu = 0.35$, pH 7.1.

the technique of Perrone (8), as modified by Padieu (9) and Kruh and associates (10). Its purity was systematically checked by paper chromatography according to Biserte and Osteux (11).

Measurement of radioactivity. Myosin, like the water-soluble proteins, was precipitated by half a volume of 20 per cent trichloroacetic acid. The precipitates were washed twice with 7 per cent trichloroacetic acid, twice with acetone, and once with ether. The dry proteins were ground into a fine powder in a Potter apparatus and in an agate mortar, and then spread in a regular layer of 40 mg protein on copper planchets of 1.52 cm^2 surface, which constitutes infinite thickness. The DNP-glycine was dissolved in methanol and estimation was made in a Beckman DU spectrophotometer at 360 mμ on an aliquot, after dilution in 2 per cent $NaHCO_3$. The radioactivity was measured on another aliquot after heat dessication on a copper planchet of 2.85 cm^2 surface in which a lens paper was fixed with silicone grease. A Geiger-Müller counter with a thin end-window was used, and the specific activity of DNP-glycine was expressed in counts per minute per millimole; the correction for thickness was made. The determinations were carried out with a statistical error of less than ± 2 per cent.

RESULTS

Normal mice (strain RAP)

Myosin (Figure 2). Two series of experiments involving 40 mice showed a constancy of radioactivity between the second and twentieth days with a mean value of 30 cpm ± SD = 2. Then the radioactivity decreased and remained constant until the thirty-eighth day at a level of 19 ± SD = 2.5.[1]

Water-extractable proteins (Figure 2). The radioactivity of the water-extractable proteins was much higher than that of myosin on the second day, then it decreased exponentially.

[1] $SD = \sqrt{\frac{(x - x_0)^2}{n - 1}}$.

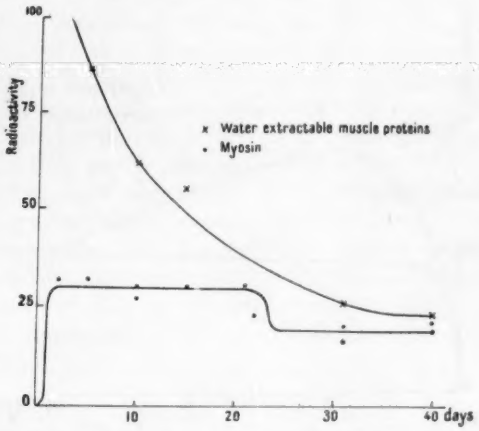

FIG. 2. RADIOACTIVITY OF MYOSIN AND WATER-EXTRACTABLE PROTEINS, MOUSE STRAIN RAP. The radioactivities are measured on 40 mg of protein on planchets of 1.52 cm^2 surface and expressed in counts per minute.

Free glycine of muscle (Figure 5). The specific activity of free glycine decreased exponentially from the second day.

Dystrophic mice and normal litter mate (strain 129)

The mice were killed at the sixth hour and on the first, second, fourth, sixth and fifteenth days after the injection of C^{14}-glycine.

Myosin (Figure 3). In the normal litter mate the radioactivity was constant between the first and the fifteenth days. In the dystrophic mice the radioactivity increased until the fourth day, when it was twice as high as that in the normal mice. After the sixth day it decreased, and on the fifteenth day the value of the radioactivity was close to that of the normal mice.

Water-extractable proteins (Figure 4). In normal as well as in dystrophic mice the radioactivity increased until the twenty-fourth hour and then decreased, but the variation as a function of time was much larger in the dystrophic than in the normal mice. The ratio of the radioactivities was 2 in the twenty-fourth hour and 1.3 on the fifteenth day.

Free glycine of muscle (Figure 5). The specific activity of free glycine decreased exponentially until the first or second day, then the slope of the semilogarithmic curve changed. The de-

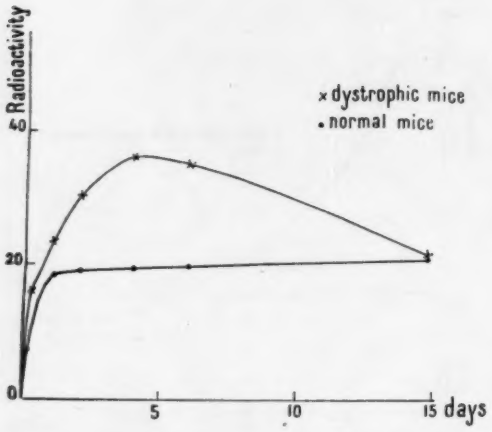

FIG. 3. RADIOACTIVITY OF MYOSIN. The radioactivities are measured on 40 mg of protein on planchets of 1.52 cm² surface and expressed in counts per minute.

crease, however, remained exponential until the fifteenth day, confirming the results previously obtained (12). The curves of the specific activity were approximately the same in the normal and in the dystrophic mice.

DISCUSSION

This work on normal mice confirms the results obtained with rats (5, 6). Myosin does not show any apparent turnover in mice on a normal protein diet. The constancy of the radioactivity seems to substantiate a life span of the myofibrils of 30 days in the rat, and 20 days in the mouse, according to the present work. After the decline of the specific activity at about the twenty-second day, the radioactivity of the myosin presents a second plateau which could be easily explained by the re-utilization of the labeled amino acids liberated by the degradation of the radioactive myosin; such a re-utilization has been shown to occur with radioactive iron in hemoglobin. Water-extractable proteins show an exponential decrease, which means that the sarcoplasmic proteins are subject to turnover.

The acceleration of protein metabolism in dystrophic mice has been demonstrated by Simon, Lessell, Gross and Milhorat (13). These authors injected C^{14}-leucine into mice and observed a faster incorporation of the amino acids into the proteins and a faster rate of disappearance from the proteins in the dystrophic animals. The more recent knowledge of the myofibrils' life span and the isolation of pure myosin provide some information on the localization of the biochemical lesion. The lesion may be localized at a molecular, a subcellular, or a cellular level. The difference in behavior cannot be explained by a modification of the permeability of the muscle cell to glycine, since we have not found any significant difference between the specific activity of free glycine in normal and in dystrophic mice. Nevertheless, a difference of penetration of radioactive glycine into the cells in the first hours after the injection cannot be excluded. It would explain the more rapid increase of radioactivity but not the more rapid subsequent decrease.

At a molecular level results could be partly explained by an acceleration of muscle protein turnover according to the hypothesis of Simon and coworkers (13). We have previously shown that in rats submitted to a high protein diet, myosin shows a slow turnover which, in rats submitted to a standard diet, was disguised because of the reutilization of radioactive glycine. In rats submitted to such a diet the radioactivity of myosin declined progressively. In pathological conditions, with accelerated turnover of myosin, the turnover can become apparent even with the standard diet. The finding of an acceleration of the turnover of the water-extractable muscle proteins is in keeping with a general acceleration of the turnover of

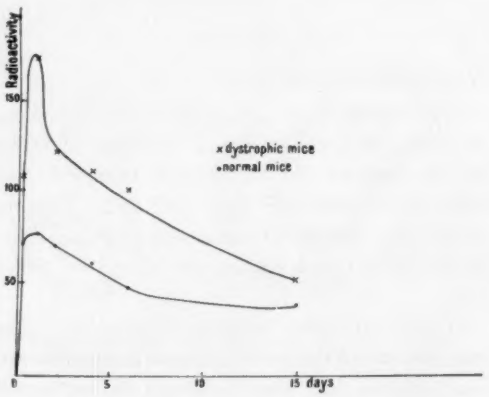

FIG. 4. RADIOACTIVITY OF WATER-EXTRACTABLE PROTEINS. The radioactivities are measured on 40 mg of protein on planchets of 1.52 cm² surface and expressed in counts per minute.

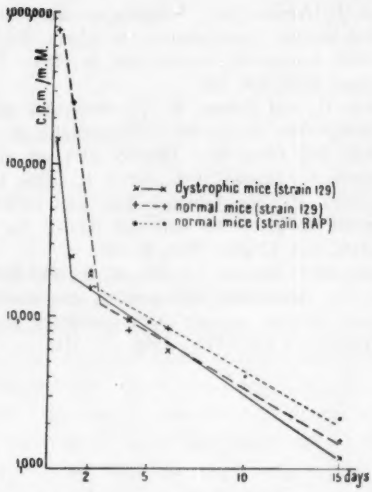

FIG. 5. SPECIFIC ACTIVITY OF FREE GLYCINE OF MUSCLE. The specific activities are expressed in counts per minute per millimole of DNP-glycine.

all the muscle proteins. However, an acceleration of the turnover should lead to a faster rate of incorporation of the labeled glycine into myosin. Figure 3 shows that in dystrophic mice there is a rise in the radioactivity of myosin during the first four days instead of for one day as in normal mice. A possible explanation of this discrepancy might be the release of amino acids derived from the degradation of radioactive water-extractable proteins and their utilization inside the cell for the synthesis of new myosin molecules.

At the subcellular level, these results could be interpreted by a shorter life span or by the absence of a life span of myofibrils, as in the case of the red blood cells in hemolytic and sickle cell anemia. This hypothesis, however, would only explain the acceleration of the myosin turnover. Due to the relatively small number of experiments, it would be impossible to make any distinction between an absence of a life span of the myofibrils and a shorter life span of these particles which would involve a short plateau.

At the cellular level, the results could be explained by a shorter life span of the muscle cells, with an acceleration of the formation of new cells. Michelson and associates (1) have seen, in dystrophic mice, division of muscle fibers into two or three parts. A more detailed study is still needed since the life span of the muscle cell has not yet been established. It seems that the results presented here could be explained by an acceleration of the turnover of muscle proteins or by a shorter life span of the muscle cells, or by both hypotheses. It is not possible at present to determine which of these two hypotheses may be correct, but the first seems more likely. An acceleration of the turnover of the muscle proteins is probably the most important abnormality of muscle protein metabolism in dystrophic mice. It would be important to know whether or not the same abnormality occurs in human muscular dystrophy.

SUMMARY

The authors have studied the metabolism of myosin and water-extractable proteins in normal mice and in mice with hereditary muscular dystrophy.

1. In normal mice the constancy of the radioactivity of myosin favors the interpretation of a life span for the myofibrils of approximately 20 days. The radioactivity of the water-extractable proteins decreases exponentially, due to a turnover of these proteins. These results are in agreement with those obtained previously in rats.

2. In dystrophic mice the radioactivity of myosin increases for the first four days, then decreases without a plateau. The turnover of the water-extractable muscle proteins is highly accelerated. These results can be interpreted as representing a shorter life span of the muscle cell, or of a part of the cell or, more likely, as an acceleration of the turnover of the muscle proteins.

REFERENCES

1. Michelson, A. M., Russell, E. S., and Harman, P. J. Dystrophia muscularis, a hereditary primary myopathy in the house mouse. Proc. nat. Acad. Sci. (Wash.) 1955, **41**, 1079.
2. Stevens, L. C., Russell, E. S., and Southard, J. L. Evidence on inheritance of muscular dystrophy in an inbred strain of mice using ovarian transplantation. Proc. Soc. exp. Biol. (N. Y.) 1957, **95**, 161.
3. Shemin, D., and Rittenberg, D. Life span of human red blood cell. J. biol. Chem. 1946, **166**, 627.
4. Neuberger, A., Perrone, J. C., and Slack, H. G. B. The relative metabolic inertia of tendon collagen in the rat. Biochem. J. 1951, **49**, 199.

5. Dreyfus, J. C., Kruh, J., and Schapira, G. Metabolism of myosin and life time of myofibrils. Biochem. J. In press.
6. Dreyfus, J. C., Kruh, J., and Schapira, G. Metabolisme des protéines musculaires *in* International Conference on Radioisotopes in Scientific Research. New York, Pergamon Press, 1958, vol. III, p. 386.
7. Szent-Györgyi, A. G. A new method for the preparation of actin. J. biol. Chem. 1951, **192**, 361.
8. Perrone, J. C. Separation of amino-acids as dinitrophenyl derivatives. Nature (Lond.) 1951, **167**, 513.
9. Padieu, P. Biosynthèse des protéines musculaires et section du nerf moteur. Bull. Soc. Chim. biol. (Paris) 1959, **41**, 57.
10. Kruh, J., Dreyfus, J. C., Schapira, G., and Padieu, P. Non-uniform incorporation of glycine-2-C^{14} into rabbit hemoglobin *in vivo* and *in vitro*. J. biol. Chem. 1957, **228**, 113.
11. Biserte, G., and Osteux, R. La chromatographie de partage sur papier de dinitrophényl-aminoacides. Bull. Soc. Chim. biol. (Paris) 1951, **33**, 50.
12. Schapira, G., Dreyfus, J. C., Kruh, J., Labie, D., and Padieu, P. Sur l'absence d'un pool métabolique unique du glycocolle libre des tissus. Bull. Soc. Chim. biol. (Paris) 1959, **41**, 469.
13. Simon, E. J., Lessell, I., Gross, C. S., and Milhorat, A. T. Amino-acid incorporation into muscle and liver proteins of mice with hereditary muscular dystrophy. Fed. Proc. 1958, **17**, 311.

THE FATTY ACID COMPOSITION OF HUMAN THORACIC DUCT LYMPH LIPIDS *

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Fernandes, Van de Kamer and Weijers (1) in studying a child with chylothorax found that the pattern of the fatty acids in the chyle was influenced by the kind of fat fed, but significant differences between chyle and dietary fat always remained. Recent experiments with carboxyl-labeled fatty acids by Blomstrand and co-workers (2–4) indicate that the chyle triglycerides constitute the main transport form of ingested fatty acids in man. Detailed information about the influence of dietary fatty acids on the fatty acid composition of the different lipid classes of human chyle is, however, still lacking. Furthermore, in order to effectively evaluate studies on the post-absorptive metabolism of chylomicron fatty acids, it is necessary to ascertain the nature of the fatty acid components of the various chyle lipids of thoracic duct lymph.

Development of methods for obtaining thoracic duct lymph from human beings (5) and the analysis by gas-liquid chromatography (GLC) of the different lipids of human chyle after feeding a well defined triglyceride has made it possible to study the relationship between the fatty acid patterns of the different lymph lipid classes and the dietary fat.

EXPERIMENTAL

Experimental design. Patient A of this study was a 56 year old man with inoperable adenocarcinoma of the left lung. He was in good condition during the whole investigation. He was nourished for 6 days solely by an orally administered liquid formula (Table I) supplemented with vitamins and minerals (6). The daily intake of formula was divided into 5 equal portions fed at 7 and 10 a.m. and at 1, 4 and 7 p.m. Tea or coffee was offered *ad libitum*. The fat in the formula diet consisted of a sterol-free triglyceride with fatty acid composition according to Table II.

On the third day of the experimental period the thoracic duct was cannulated as described earlier (5) and the lymph was collected for a 10 minute period every hour during the next 36 hours. After that time the catheter was withdrawn without any untoward effect. On the fourth day of the experiment the first formula was fed at 7 a.m. and the rest of the day the formula was fed every third hour.

The lymph was collected for 10-minute periods in ethanol to prevent any enzymatic hydrolysis during the collection time. The feces were collected in ethanol and stored in an icebox until analyzed.

Patient B of this study was a 60 year old man with adenocarcinoma of the right lung. He was in good condition during the investigation and was fed a general mixed diet. The lymph sample analyzed was collected from the thoracic duct during the peak of fat absorption after feeding a fat-rich breakfast.

Extraction and fractionation of lymph lipids. The total lipids of the lymph were extracted with 20 volumes of ethanol: ether, 3:1. The solvent was evaporated *in vacuo*, and the total lipids were taken up in petroleum ether (bp 60 to 70°C) and made up to volume. Aliquots were subjected to chromatography on silicic acid in order to obtain a neutral fat free fatty acid fraction and a phospholipid fraction (7, 8). The free fatty acids were then removed from the neutral fat by extraction with alkaline 50 per cent ethanol and re-extraction of the fatty acids with light petroleum after acidification. The neutral fat fraction (glycerides, cholesterol and cholesterol esters) were subjected to chromatography on silicic acid for separation of cholesterol esters and a triglyceride + cholesterol fraction (Table III).

TABLE I

*Composition of liquid formula diet fed daily as sole source of nutrients to Patient A**

Constituents	Wt.
	g
Triglycerides, sterol-free	210
Milk protein	470
Dextrose	670
Water	5,000

* The patient was fed 300 ml five times a day; 1,500 ml of this formula contains approximately 1,900 calories.

* This work is part of the investigations supported by the Swedish Medical Research Council and the Scientific Council of the Swedish Dairies Association.

TABLE II

*The fatty acid patterns in human thoracic duct lymph lipids after feeding a well defined triglyceride in a liquid formula diet (Patient A) and sampling of the thoracic duct lymph during active fat absorption**

Fatty acid		Triglyceride fed	Thoracic duct lymph lipids				Fecal FA
			TG	FFA	Ch.E.	PL	
		%	%	%	%	%	%
Saturated							
Lauric	12:0			3.6	0.3		
Myristic	14:0	1.0	0.5	3.4	0.1	0.4	1.2
Pentadecanoic	15:0	0.1		4.0		0.4	2.0
Palmitic	16:0	18.3	16.4	20.3	21.6	23.1	20.2
Heptadecanoic	17:0	0.3 ?		9.2	0.2	0.3	1.0
Stearic	18:0	3.0	2.2	5.7	6.5	14.6	13.0
Monounsaturated							
Palmitoleic	16:1	2.4	2.7	12.2	1.7	1.1	4.3
Oleic	18:1	74.2	70.4	19.5	58.7	32.4	48.2
Diunsaturated							
Linoleic	18:2	0.5	7.7	16.0	10.8	21.3	4.3
Polyunsaturated							
Linolenic	18:3					0.5	
Arachidonic	20:4					3.0	
	20–22?					2.4	2.8
Sum†		99.8	99.9	93.9	99.9	99.5	97.0

* TG = triglyceride fatty acids, FFA = free fatty acids, Ch.E. = cholesterol ester fatty acids, PL = phospholipid fatty acids. The component fatty acids are also designated by a dual symbol giving chain length and number of double bonds. Values are expressed as percentage of total fatty acid methyl esters.

† The sum of each column is less than 100 per cent because trace amounts of unusual fatty acids detected were omitted from the final tabulation.

The lipids in aliquots of the test meals were extracted by the same method as that described for extraction of lymph lipids. Extraction was also made on the daily amount of wet feces with alcohol and ether as described earlier (9). The different lipid fractions were saponified and the fatty acids isolated as described previously (2). Methyl esters of the fatty acids were made by treating the fraction with a solution of diazomethane in ether. After standing, the ether and the diazomethane were carefully evaporated on the water bath. The methyl esters were dissolved in petroleum ether and stored in a refrigerator until analyzed.

The phospholipids were fractionated on silicic acid by the procedure described by Hanahan, Dittmer and Warashina (10). The loading ratio was 0.5 mg of lipid phosphorus per g of silicic acid and the usual solvents were chloroform-methanol mixtures (4:1 and 1:4, vol/vol, respectively). The progress of the chromatographic fractionation was followed by phosphorus assay and paper chromatography. The fractions containing lecithin were further rechromatographed on aluminium oxide (4) because the pure lecithin thus obtained reacted more satisfactorily with phospholipase A (*Crotalus adamanteus* venom).

Lecithin prepared from the lymph lipids of Patient A (Table IV) (7.0 mg of lipid P) was dissolved in 100 ml of diethyl ether and treated with 2 mg of snake venom (*Crotalus adamanteus*) in 1 ml of aqueous 0.1 M calcium chloride solution. A fine stream of anhydrous ammonia was bubbled through the ether solution (11). A considerable amount of precipitate formed within two hours, and after four hours the solvent was removed by evaporation and reduced pressure. The residue was dissolved in chloroform and applied to a column of 5 g of silicic acid. Elution was continued with chloroform until all fatty acids released by phospholipase A had been obtained. After removal of the solvent the methyl esters were prepared with diazomethane. Next, the column was eluted with chloroform: methanol 4:1 and 1:4, respectively, in order to separate unchanged lecithin from lysolecithin. The fatty acids of the lysolecithin were obtained after hydrolysis, acidification and extraction with light petroleum.

Analysis of fatty acid. GLC was carried out essentially as described by James and Martin (12) using an Argon Pye chromatograph with an ionization chamber as detector. The mobile phase (argon) was applied at a pressure of 72 cm Hg. Each four foot column was packed with acid-washed, alkali-treated celite 545 (mesh 120 to 140). As stationary phase, a polar polyester (LHC-1R-296, obtained from Cambridge, Mass.) was used in the ratio celite: stationary phase, 4:1 (13). This column had a retention time of 10 minutes for methyl stearate. The retention volumes of the normal fatty

TABLE III

*Composition of thoracic duct lymph lipids of Patient A after feeding a well defined triglyceride in a liquid formula diet and sampling of the thoracic duct lymph**

Patient	Collection time	Lymph volume per minute	Total cholesterol fat			Fatty acids as per cent of total fatty acids			
				Free	Total	Cholesterol FA	FFA	Triglyceride FA	Phospholipid FA
	min	*ml*	*g %*	*mg %*					
A	10	2.0	4.4	35	80	1.3	2.7	86.0	11.0
B	10	1.6	4.7	44	95	1.6	2.1	85.5	10.6

* The thoracic duct lymph was collected for 10-minute periods every hour during 36 hours. The lymph sample analyzed was collected at 11 a.m. on the fourth day of the experiment. Patient B was on a free diet during the collection period at 11 a.m.

acids were determined using a mixture of pure esters of fatty acids obtained from the Hormel Institute (Austin, Minn.). Major acids were identified by comparison with the retention volumes of these pure standards. For identification the samples were also chromatographed at 240°C on a column with silicone oil (Dow-Corning high vacuum grease) as stationary phase in the ratio celite : silicone oil, 4 : 1. For determination of branched and unsaturated fatty acid, the samples were also run after hydrogenation.

In this report fatty acids are numbered as suggested by Insull, Hirsch, James and Ahrens (14). Thus 18 : 0 refers to the straight chain saturated C_{18} acid, stearic, 18 : 2 = C_{18}-diene and so forth.

Results were computed by the triangulation method.

RESULTS

Table III permits comparison of the analytical data for the dietary triglyceride fatty acids with the figures for the different lipid classes of the thoracic duct lymph. The lymph sample analyzed was collected four hours after feeding the first test meal at 7 a.m. on the fourth day of the experiment. The fecal fatty acids are from the stools collected on the fifth day of the experimental period. Data derived from analysis of fecal lipids are also shown.

Lymph cholesterol esters. The fatty acids found in the ester linkage with a lymph cholesterol were chiefly oleic acid, 58.7 per cent, and palmitic acid, 21.6 per cent. The concentrations of these fatty acids are very similar in the dietary triglyceride and the lymph cholesterol esters. Stearic acid, 6.5 per cent, and linoleic acid, 19.8 per cent, were both found in higher concentration than in the dietary triglyceride.

Lymph triglycerides. The fatty acid composition of the lymph triglycerides is almost exactly the same as that in the fed triglyceride. Oleic acid, constituting 74.4 per cent of the fed triglyceride, is found in the high concentration of 70.2 per cent in the lymph triglycerides. Palmitic and stearic acid also have almost the same concentration in the lymph triglycerides as in the diet. A remarkable difference is the higher concentration of linoleic acid, 7.7 per cent in the lymph triglycerides compared with 0.5 per cent in the diet.

Phospholipid fatty acids. The composition of the phospholipid fatty acids is different from that of the dietary triglycerides, and the triglycerides and the cholesterol esters of the lymph. The major acids are oleic acid, 32.4, stearic acid, 14.6, and linoleic acid, 21.3 per cent. The major component of the lymph phospholipids is lecithin. A similar fatty acid composition has been described earlier for human lecithin on a general mixed diet (4).

In Experiment A the lymph lecithin was isolated and the fatty acids freed by phospholipase A were determined with GLC (Table IV). In this fraction 85 per cent of the total fatty acids were unsaturated fatty acids. On the other hand, in the fatty acids isolated from the lysolecithin, 67 per cent of the total fatty acids were saturated. In the fatty acids released by phospholipase A, the three major fatty acids were palmitic, 15.9, oleic, 24.2, and linoleic acid, 34.7 per cent. Small amounts of myristic, pentadecanoic and stearic acid were also present, as well as palmitoleic, linolenic and arachidonic acid.

Two fatty acids with 20 to 22 carbon atoms were present on the gas chromatogram. In the lysolecithin the three major fatty acids were palmitic, 33.2 stearic 26.8 and oleic acid, 25.2 per

TABLE IV

*The fatty acid composition of human thoracic duct lymph lecithin (Patient A) after feeding a well defined triglyceride in a liquid formula diet**

Fatty acid		Percentage of total fatty acids in respective positions of human lymph lecithin	
		Fatty acids freed from lymph lecithin by phospholipase A	Fatty acids obtained after hydrolysis of lysolecithin
Saturated			
Myristic	14:0	0.7	0.8
Pentadecanoic	15:0	0.5	0.8
Palmitic	16:0	15.9	33.1
Heptadecanoic	17:0	0.3	0.8
Stearic	18:0	8.6	26.8
Monounsaturated			
Palmitoleic	16:1	1.6	4.7
Oleic	18:1	24.2	25.2
Diunsaturated			
Linoleic	18:2	34.7	3.7
Polyunsaturated			
Linolenic	18:3	1.0	
Arachidonic	20:4	6.1	
	20–22?	4.4	
Sum†		98.0	95.9

* The lecithin was purified by silicic acid column chromatography and hydrolyzed with phospholipase A. For explanation of symbols see Table II.

† The sum of each column is less than 100 per cent because trace amounts of unusual fatty acids detected were omitted from the final tabulation.

cent: Small amounts of palmitoleic and linoleic acid were also present.

The composition of the lymph phospholipid fatty acids is apparently not influenced to any high degree by the fed dietary fatty acids.

Free fatty acids (FFA). This fraction was obtained from the human thoracic duct lymph after isolation of a neutral fat plus FFA fraction from the phospholipids on silicic acid. The FFA were then extracted with alkaline 50 per cent ethanol and recovered into light petroleum after acidification.

The major FFA in both experiments was palmitic acid comprising 20.3 and 24.6 per cent, respectively. In Experiment A oleic acid constituted only 19.5 per cent compared with 74.2 per cent in the diet. An interesting finding was the relatively high percentage of palmitoleic acid in both experiments, 12.2 and 18.6, respectively. In Experiment B there was more palmitoleic acid than oleic acid in the FFA. In both experiments there was a clear tendency to higher concentrations of fatty acids with 10 to 15 carbon atoms in the free fraction.

In Table V the results of the analyses of the lymph lipids obtained from Patient B are shown. This patient was fed a general mixed diet during the whole experimental period. From the analytical data in Table V it is apparent that the three lymph lipid classes analyzed have three distinctly different fatty acid patterns.

The three major components of the lymph triglycerides are myristic, palmitic and oleic acid. Oleic acid is, however, not so predominant as in the experiment with Patient A, in which the major component in the fed triglyceride was oleic acid.

The general picture of fatty acid pattern of the lymph phospholipids was similar in the two patients, but there were slight differences in the percentages of some of the components present. In particular, the lymph phospholipids of Patient B contained more linoleic acid.

Regarding the fecal fatty acids analyzed from Patient A, significant differences can be found between the fed composition of the fed fatty acids and the fecal fatty acids. The diet was very well absorbed and from earlier experiments (1) it can be expected that about 50 per cent of the fecal

TABLE V

*The fatty acid patterns in human thoracic duct lymph lipids of Patient B after feeding a general mixed diet and sampling of the thoracic duct lymph during active fat absorption**

Fatty acid		Thoracic duct lymph lipids			
		TG	FFA	Ch.E.	PL
		%	%	%	%
Saturated					
Decanoic	10:0		1.4		
Undecanoic	11:0		1.1		
Lauric	12:0	2.8	2.6		2.2
Myristic	14:0	11.9	5.0		7.3
Pentadecanoic	15:0	1.8	2.0		1.2
Palmitic	16:0	24.6	24.6		35.4
Heptadecanoic	17:0	1.0	2.7		
Stearic	18:0	6.1	8.8		13.3
Monounsaturated					
Myristoleic	14:1		4.7		
Palmitoleic	16:1	5.0	18.6		6.5
Oleic	18:1	31.6	16.1		25.1
	18:1		4.2		
Diunsaturated					
Linoleic	18:2	8.6	6.5		6.5
Polyunsaturated					
Linolenic	18:3	0.8			
	20–22?	1.8			
Sum†		96.0	98.3		97.5

* For explanation of symbols see Table II. Values are expressed as percentage of total fatty acid methyl esters.
† The sum of each column is less than 100 per cent because trace amounts of unusual fatty acids detected were omitted from the final tabulation.

fatty acids are of dietary origin. The fecal fatty acids are somewhat more saturated and they contain significantly more stearic acid, which may in part originate from hydrogenated oleic acid in the diet (15). Especially interesting is the presence of fatty acids with 20 to 22 carbon atoms. The exact nature of these fatty acids has not yet been determined. Analyses of human bile (16) have revealed the presence of fatty acids belonging to the C_{20} and C_{22} groups in the bile lipids.

DISCUSSION

Although the fatty acid composition of the total fat of human chyle was determined earlier (1), there has been little information reported until recently (4) on the composition of the individual lipids of human thoracic duct lymph. Dole and associates (17) have reported the fatty acid composition of human chylomicra in plasma during fasting and alimentary lipemia. It was found that neither the chylomicra nor any other fractions acquired the pattern of dietary fat during the period of lipemia. As is apparent from the results of this investigation (Table II) the fatty acids of the lymph triglycerides acquired very rapidly the patterns of dietary fat during the absorption process. The triglyceride fatty acids of human chyle constitute approximately 85 per cent of the total fatty acids of chyle, and the relative stability of the plasma fatty acid reported during absorption (17) must depend on a rapid removal of the chylomicron fatty acids, presumably by the liver, and recycling of fatty acids between blood and tissue pools.

It is of interest to note that the FFA pattern of the human thoracic duct lymph did not follow changes in the triglyceride pattern, nor was it influenced by the fed fat. Apparently this fraction was not derived from lipolysis of the lymph triglycerides. This fraction may represent the sum of albumin-bound FFA originating from serum as well as FFA derived from the fat depots drained by the lymph. These results also confirm other experiments in rats (18) and in man (2).

The fatty acid composition of the cholesterol esters was highly influenced by the dietary tri-

glyceride as shown by the high percentage of oleic acid (Table II). Apparently dietary fatty acids are used for esterification of cholesterol during the absorption process. In view of recent studies of the effects of unsaturated and saturated fatty acids on serum cholesterol levels (19) and the demonstration that cholesterol excretion by intestine is in part controlled by the composition of the dietary fat (20), the finding in this investigation of a dietary effect on the fatty acid composition of the lymph cholesterol esters might have some bearing on the direct control of the absorption of cholesterol. Different fatty acids might influence the absorption of endogenous and dietary cholesterol from the intestine in such a significant way that the effects of dietary fatty acids on the serum cholesterol might be mainly an absorption effect. It will be of interest to continue these studies with comparison of variously constituted dietary fats on the absorption of cholesterol in patients with thoracic duct fistula.

In the lymph phospholipids the fatty acid composition was not influenced to any significant degree by the dietary fat. The fatty acid composition of the lymph phospholipids was in fact very similar in the two experiments, in spite of the different diet fed. Most of the phospholipids of the lymph represent endogenous phospholipids in agreement with previous experiments in animals (21) and in man (2, 4).

According to a recent work by Tattrie (22) the site of the attack of phospholipase A should be at the C-2 (beta) position instead of at the C-1 (alpha) position as has been proposed by Hanahan (23). According to this new concept the unsaturated fatty acids predominated in the C-2 (beta) ester position, although saturated fatty acids such as myristic, palmitic and stearic acid were also isolated from this position. In the C-1 (alpha) position saturated fatty acids predominated but oleic acid constituted the same percentage of the total fatty acids in both positions (4). A small amount of palmitoleic and linoleic acid was also obtained in the C-1 position. Apparently there is an asymmetrical distribution of many fatty acids in the human lymph lecithins. This asymmetrical distribution is, however, not obligatory, as can be seen from the finding that oleic acid is distributed almost equally between the C-1 (alpha) and the C-2 (beta) positions. A similar distribution pattern for oleic acid has recently been obtained in liver lecithins of rat (24).

Apparently there are several different lecithins present in the lymph and for a more explicit understanding of their metabolism it is necessary to isolate the individual species. The pronounced positional asymmetry of the fatty acids of the lecithin molecules of the lymph also indicates that for complete elucidation of the metabolic pathways of the different fatty acids during the absorption process the positional location of the individual fatty acid on both phospholipids and triglycerides must be considered.

SUMMARY

1. Analyses were made of the fatty acid composition of the lipid components of human thoracic duct lymph by gas liquid chromatography.

2. The fatty acid distribution in the lymph triglycerides and the cholesterol esters was largely influenced by the dietary fat. The free fatty acids had quite another pattern than did the lymph triglycerides and were not influenced by the dietary fat.

3. The phospholipid fatty acids were influenced very little by the dietary fat. An asymmetrical distribution of the fatty acids of the lymph lecithins uninfluenced by the fat fed is described.

REFERENCES

1. Fernandes, J., Van de Kamer, J. H., and Weijers, H. A. The absorption of fats studied in a child with chylothorax. J. clin. Invest. 1955, **24**, 1026.
2. Blomstrand, R., and Ahrens, E. H., Jr. The absorption of fats studied in a patient with chyluria. J. biol. Chem. 1958, **233**, 321.
3. Blomstrand, R., Dahlbäck, O., and Linder, E. Asymmetric incorporation of linoleic acid-1-C^{14} and stearic acid-1-C^{14} into human lymph lecithins during fat absorption. Proc. Soc. exp. Biol. (N. Y). 1959, **100**, 768.
4. Blomstrand, R., and Dahlbäck, O. Gas-liquid chromatography of human lymph fatty acids after feeding C^{14}-labelled fats. Acta Soc. Med. upsalien. 1959, **64**, 177.
5. Linder, E., and Blomstrand, R. Technic for collection of thoracic duct lymph of man. Proc. Soc. exp. Biol. (N. Y.) 1959, **97**, 653.
6. Ahrens, E. H., Jr., Dole, V. P., and Blankenhorn, D. H. The use of orally-fed liquid formulas in metabolic studies. Amer. J. clin. Nutr. 1954, **2**, 336.
7. Borgström, B. Investigation on lipid separation

method. Separation of phospholipids from neutral fat and fatty acids. Acta physiol. scand. 1952, **25**, 102.

8. Borgström, B. Investigation on lipid separation methods. Separation of cholesterol esters, glycerides and free fatty acids. Acta physiol. scand. 1952, **25**, 111.
9. Blomstrand, R. A study on the intestinal absorption of fat in normal adults and in non-tropical sprue with carbon-labelled oleic and palmitic acid. Acta med. scand. 1955, **152**, 1.
10. Hanahan, D. J., Dittmer, J. C., and Warashina, E. A column chromatographic separation of classes of phospholipides. J. biol. Chem. 1957, **228**, 685.
11. Long, C., and Penny, I. F. The structure of naturally occurring phosphoglycerides. III. Action of moccasin-venom phospholipase A on ovolecithin and related substances. Biochem. J. 1957, **65**, 382.
12. James, A. T., and Martin, A. J. P. Gas-liquid partition chromatography: The separation and microestimation of volatile fatty acids from formic acid to duodeconic acid. Biochem. J. 1952, **50**, 679.
13. Orr, C. H., and Callen, J. E. Recent advances in the gas chromatographic methyl esters of fatty acids. Ann. N. Y. Acad. Sci. 1959, **72**, 649.
14. Insull, W., Jr., Hirsch, J., James, T., and Ahrens, E. H., Jr. The fatty acids of human milk. II. Alterations produced by manipulation of caloric balance and exchange of dietary fats. J. clin. Invest. 1959, **38**, 443.
15. Bergström, S., and Blomstrand, R. Studies on the intestinal absorption of fat in man with the aid of labelled oleic and palmitic acid *in* Biochemical Problems of Lipids (Proc. Soc. Int. Conf. Ghent, 1955). London, Butterworth, 1956, p. 323.
16. Blomstrand, R. Analysis of human bile lipids by gas-liquid chromatography. Acta chem. scand. In press.
17. Dole, V. P., James, A. T., Webb, J. P. W., Rizack, M. A., and Sturman, M. F. The fatty acid patterns of plasma lipids during alimentary lipemia. J. clin. Invest. 1959, **38**, 1544.
18. Borgström, B., and Tryding, N. Free fatty acid content of rat thoracic duct lymph during fat absorption. Acta physiol. scand. 1956, **37**, 127.
19. Ahrens, E. H., Jr., Insull, W., Jr., Blomstrand, R., Hirsch, J., Tsaltas, T., and Peterson, M. L. The influence of dietary fats on serum lipid levels in man. Lancet 1957, **272**, 943.
20. Hellman, L., Rosenfeld, R. S., Insull, W., Jr., and Ahrens, E. H., Jr. Intestinal excretion of cholesterol: A mechanism for regulation of plasma levels (abstract). J. clin. Invest. 1957, **36**, 898.
21. Bergström, S., Blomstrand, R., and Borgström, B. Route of absorption and distribution of oleic acid and triolein in the rat. Biochem. J. 1954, **58**, 600.
22. Tattrie, N. H. Positional distribution of saturated and unsaturated fatty acids on egg lecithin. J. Lip. Res. 1959, **1**, 60.
23. Hanahan, D. J. The site of action of lecithinase A of lecithin. J. biol. Chem. 1954, **207**, 879.
24. Dittmer, J. C., and Hanahan, D. J. Biochemistry of long chain fatty acids. 1. General observations. J. biol. Chem. 1959, **234**, 1976.

DETECTION IN NORMAL URINE OF PROTEIN RESEMBLING BENCE JONES PROTEIN

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Bence Jones (1), in 1848, described an unusual protein present in high concentration in the urine of a patient suffering from multiple myeloma. Similar urinary proteins have subsequently been found in many cases of this disease, and are designated by the collective term "Bence Jones protein." They are characterized by their behavior when heated in solution, usually giving two identifying reactions if present in sufficient concentration (2, 3): *a*) with the pH near 5, precipitation occurs between 45° and 60° C; *b*) subsequent heating to boiling causes complete or partial solution of the precipitate, which re-forms on cooling.

Studies of other physicochemical properties have given rather diverse results. Molecular weights in the range 24,000 to 90,000 have been calculated (4), with a median about 44,000 (3). Total and N-terminal amino acid analyses have given varying results (3, 5). Despite this heterogeneity of the group as a whole, specimens from any one individual have usually tended to physical and chemical homogeneity (5).

Recent studies of antigenic structure (6, 7) suggest that all Bence Jones proteins are antigenically related to the serum gamma globulins. In addition, any individual Bence Jones protein lacks some gamma globulin determinants, but possesses determinants unique for a group of Bence Jones proteins (7) and possibly for itself alone (8).

There is lack of agreement on conditions of occurrence of this protein in urine. A positive heat test has been said to be almost pathognomonic of multiple myeloma and to be present in about 50 per cent of cases of this disease (9); however, sporadic reports occur (10, 11) of detection of the protein by this method in other diseases. Immunochemical testing is reported to detect it in practically all cases of myeloma (12), and in some cases of other diseases (13).

This paper describes attempts to detect Bence Jones protein by immunochemical means in normal serum and urine. An antiserum to gamma globulins [1] was used. Any protein detected by its reaction with this antiserum must be further characterized in order to distinguish gamma globulins, Bence Jones protein, and any other gamma-related protein. Criteria for such characterization were: heat stability at 55° C, solubility of heated products at 98° C, and diffusion coefficient.

The main investigation consists of studies of solutions of serum and urine protein, and of filtrates obtained from these at 55° and 98° C. Two preliminary experiments on gamma globulins and Bence Jones proteins were thought necessary to assess rates of denaturation at 55° C, solubility of denatured products at 98° C, and antigenic activity [2] of protein remaining in solution after heating at these temperatures.

MATERIALS AND METHODS

The protein solvent used extensively in this work consisted of 0.15 M NaCl–0.2 M, pH 5, sodium acetate buffer, 4:1 vol/vol; this is subsequently referred to as

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[1] The antiserum was prepared to Cohn Fraction II, which contains about 80 per cent of the plasma gamma globulins. These globulins also occur in Fraction III-1 (14), which was therefore included in physicochemical studies. The gamma globulins have a predominant component of sedimentation rate 7S and a minor component of 19S; these show some antigenic differences (15).

Protein antigenically related to the gamma globulins, i.e., reacting with antibodies prepared to them, is designated "gamma-related." Gamma-related proteins are known to include 7S and 19S gamma globulins, myeloma serum globulins, pathological macroglobulins and Bence Jones proteins (3).

An antiserum to gamma globulins can be expected to react with all Bence Jones protein (7), although some of these may be antigenically distinct from each other (7, 8), i.e., an antiserum to any single such pathological protein need not react with all others.

[2] The term "antigenic activity" is used to refer to the degree of interaction of considered proteins with a given antiserum, interaction being estimated by precipitin, precipitin-inhibition or other method.

"saline-acetate." "Sixty per cent saturated" ammonium sulfate implies 43 g of the salt per 100 ml of solvent, representing 60 per cent saturation at 2° C.

Serum gamma globulin fractions were obtained from Commonwealth Serum Laboratories, Melbourne, Australia. *Bence Jones proteins* (BJ1 to BJ6) were obtained from urines of 6 patients (selected from 18) suffering from multiple myeloma. The proteins were isolated by repeated precipitation with 60 per cent saturated ammonium sulfate.

Normal urine and serum were obtained from 7 healthy adults, A to E, males, and F and G, females. Urine was collected over a 24 hour period. After each voiding merthiolate was added to 0.01 per cent and the urine was immediately refrigerated. On completion of collection any deposits were removed by filtration, and concentration by ultrafiltration was begun immediately. When the residual volume had reached about 50 ml it was dialyzed against saline-acetate. Solid ammonium sulfate was added to 60 per cent saturation and the mixture equilibrated overnight at 2° C. The precipitate was packed by centrifugation and, if not used immediately, stored under moist conditions at 2° C. Two precipitates from Subject A contained 8.2 and 9.0 mg of nondialyzable nitrogen. Serum was obtained during the urine collection period. One ml was diluted to 10 ml with saline-acetate and the protein was precipitated and stored as detailed for the urine concentrates.

For use in Experiment 3 precipitates were dispersed in saline-acetate: those from urine in 1 ml, those from serum in 5 ml (but sometimes in different volumes, as indicated in the tabulated results). Any substance not dissolved after 2 hours at 37° C was removed by centrifugation. Serum precipitates dissolved readily with negligible insoluble residue. Urine precipitates dissolved incompletely, with a dark brown to black residuum usually containing less than 0.5 mg of nondialyzable nitrogen; the solutions were of deep brown color with a strong uriniferous odor. Proteins finally obtained in solution are subsequently referred to as "globulins."

Ultrafiltration was performed by a modification of the method described by Grant (16). The sample was filtered through 4 feet of 24/32 inch Visking dialysis casing held linearly inside a 3 inch diameter Perspex tube, a vacuum of 720 mm Hg being maintained outside the casing. Stretching of the casing was prevented by enclosing it in a steel mesh tube of the same diameter. Concentration of 1 L of urine to 50 ml required about 30 hours. Of 100 mg of Bence Jones protein dissolved in 1 L of buffered saline, over 95 per cent remained after concentration to 45 ml.

Protein quantitation was usually by a biuret method. This was inapplicable to small amounts of protein recovered from large urine volumes because of the absorbance of considerable associated pigment; in these cases nondialyzable N was determined by the Kjeldahl method. The biuret method was modified from that described by Goa (17). Protein was precipitated by trichloroacetic acid and separated by centrifugation at at least 10,000 G for 15 minutes. To the precipitate was added a 4 per cent solution of Goa's Benedict reagent in 3 per cent NaOH. The absorbance at 350 mμ was read after 15 to 60 minutes. The amount of protein was then determined from a curve constructed from gravimetric fraction II-1, 2 standards. For estimation of insoluble protein the biuret reagent was added directly to washed precipitate, which always dissolved readily.

Antisera to Fraction II were prepared in rabbits by single 4 week courses of intravenous alum-precipitated protein as recommended by Kabat and Mayer (18). Sera were decomplemented with bovine serum albumin (BSA)-antiBSA specific precipitate and stored at −30° C. Results presented in this paper were obtained by using a single pool of 6 sera. By the immunoelectrophoretic technic (19) this pool contained antibodies only to the gamma antigenic group among the serum proteins.

Precipitin tests in agar gel, using the double diffusion plate method, were performed essentially as described by Ouchterlony (20). Reservoirs were filled only once. *Quantitative precipitin tests* were porformed as detailed by Kabat and Mayer (18), except that precipitates were estimated by a biuret instead of a Kjeldahl method. Analyses of supernatants for free antigen or antibody were carried out by precipitin tests on Ouchterlony plates.

The precipitin-inhibition test consisted of incubating an excess of antiserum with the sample of unknown antigenic activity and subsequently titrating residual antibody; titration involved assessing how much Fraction II had to be added to a given volume of antiserum in order just to produce antigen excess. One ml of the unknown preparation (made up to final volume with phosphate-buffered saline to bring the pH to 7.0 ± 0.2) was incubated with 1 ml of antiserum at 37° C for 1 hour, then at 2° C for 24 hours. Precipitate was spun off and 0.2 ml lots of the supernatant were added to a series of tubes containing amounts of Fraction II in geometric progression with common ratio 1.2. The tubes were incubated and antigen excess then sought by precipitin tests on Ouchterlony plates.

Heating and filtering were standardized as follows. Solutions in narrow tubes were placed in a water bath kept at the indicated temperature; timing was begun from this moment. The tubes were stoppered after an interval allowing the contents to approach ambient temperature and were intermittently agitated. All filtrations were through Whatman no. 542 paper in an oven held within the indicated temperature range; funnels were covered to minimize evaporation. Filtering of urine globulins at 98° C had to be assisted by slight suction; the same degree of suction was applied for the serum globulins.

RESULTS

Bence Jones proteins and gamma globulins; heat stability and solubility of heated products. These results describe essentially endpoints attained in the overall reaction (dissolved protein ⇌

TABLE I

Protein solutions heated at 55 ± 1° C for 15 minutes then filtered at 55 ± 2° C for 15 minutes

	Protein		
	μg/ml	μg/ml	μg/ml
Original concentrations	5,000	1,000	200
Concentrations in filtrates of:			
BJ1	2,000	530	190
BJ2	3,500	850	230*
BJ3	1,600	510	
BJ4	1,200	380	
BJ5	1,800	600	
BJ6	1,700	710	210
Fraction II-1,2	5,100*		
Fraction II-3	5,200*		
Fraction III-1		1,050†	

* Turbidity not noted prior to filtering.
† Slight turbidity after 15 minutes, persisting in filtrates obtained at 55° C or at room temperature.

precipitated protein) on heating various preparations at 55° or 98° C. The solvent used throughout was the pH 5 saline-acetate. With heating and filtering standardized as previously described, results varied by up to 20 per cent when duplicate experiments were run at different times. The averaged results presented should therefore be taken to indicate an order of magnitude only.

TABLE II

Protein solutions heated in a boiling water bath for 5 minutes then filtered at 98 ± 4° C for 5 minutes

	Protein		
	μg/ml	μg/ml	μg/ml
Original concentrations	5,000	1,000	200
Concentrations in filtrates of:			
BJ1	1,800	1,300*	
BJ2	1,200	1,200	230*
BJ3	900	850	230*
BJ4	680	490	240*
BJ5	1,050	1,100*	
BJ6	1,400	1,300*	
Fraction II-1,2	18	<10	
Fraction II-3	28	<10	
Fraction III-1		32	<10
Fraction II + BJ2, 4:1	280†		
24:1	50‡		
Fraction II + BJ4, 4:1	205†		
24:1	55‡		

* Solution clear prior to filtering.
†,‡ Compare recoveries from solutions containing only BJ2 or BJ4 at corresponding concentrations (1,000 μg per ml and 200 μg per ml).

Table I sets forth the results of heating and filtering at 55° C for durations used in Experiment 3. Effects of varying the duration of heating were studied by removing solutions from the water bath at intervals, chilling, and centrifuging in the cold. Precipitation of most Bence Jones proteins reached a plateau at about 40 minutes, the extent of precipitation being similar to that shown in Table I; maximal precipitation of BJ2 required 160 minutes.

Table II shows the results of heating and filtering at 98° C, again for durations used in Experiment 3. Material used for the experiment depicted in Table III was obtained by heating the indicated protein at 70° C and washing the resulting precipitate with saline-acetate at room temperature; in no case was much precipitate lost in washing. The precipitates were suspended in saline-acetate, then heated and filtered at 98° C. All Bence Jones filtrates became turbid at room temperature; protein concentrations were therefore determined in the total mixtures and in second filtrates obtained at room temperature. The latter filtrates again became turbid on heating at 55° C.

TABLE III

Suspensions of washed precipitates of denatured proteins heated in a boiling water bath for 5 minutes then filtered at 98 ± 4° C for 5 minutes

Precipitate of	Total protein in filtrate	Protein remaining in solution at room temperature
	μg/ml	μg/ml
BJ1	1,000	820
BJ2	1,200	780
BJ6	1,050	900
Fraction II-1,2	<10	

Bence Jones proteins, gamma globulins and heated products; immunochemical studies. Precipitin reactions in agar plates are depicted in Figures 1 and 2. In Figure 2 one can compare the precipitate bands formed by a native Bence Jones protein (BJ6) and by a solution (BJ6dn) obtained on dissolving, at 98° C, a washed heat precipitate of the same protein. Note the absence of "spur" formation on the coalescence, the similar curvatures and the similar locations along the antiserum-antigen radii. Such a lack of distinction

between native and heated preparations was also obtained *a*) using the five other Bence Jones proteins; *b*) using antisera prepared to a Bence Jones protein (BJ1) or to a heat precipitate of this protein.

Figures 3 and 4 depict quantitative precipitin reactions of the antiserum with its homologous antigen (Fraction II), a Bence Jones protein (BJ6) and several heated preparations of BJ6.

With all six Bence Jones proteins, precipitin-inhibition tests failed to show any difference in antigenic activity between native protein and protein remaining in solution after filtering at 98° C. The small amounts of protein in 98° C filtrates of

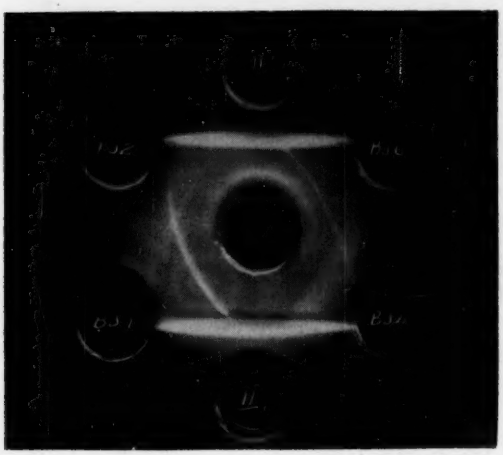

FIG. 1. AGAR PRECIPITATION PATTERNS OF ANTI-II SERUM (CENTRAL WELL) WITH FRACTION II AND BENCE JONES PROTEINS.

Fractions II and III-1 gave the same precipitin-inhibition as did the native proteins at these low concentrations.

Serum and urine globulins; immunochemical studies. Samples from A were examined on five occasions, from other subjects once. Solutions were fractionated for study of *a*) unheated solutions, filtered at room temperature; *b*) filtrates obtained by heating at 55 ± 1° C for 15 minutes, then filtered at 55 ± 2° C for 15 minutes; *c*) filtrates obtained by heating in a boiling water bath for 5 minutes, then filtered at 98 ± 4° C for 5 minutes.

Turbidity on heating at 55° C occurred as indicated in Table IV; the deep brown color of urine

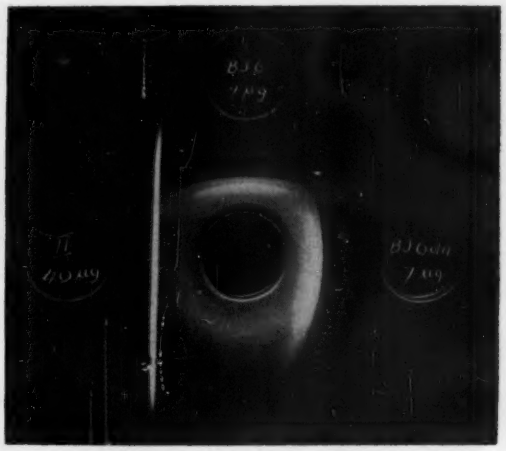

FIG. 2. AGAR PRECIPITATION PATTERNS OF ANTI-II SERUM WITH FRACTION II, A BENCE JONES PROTEIN (BJ6), AND A SOLUTION PREPARED FROM HEAT-DENATURED BENCE JONES PROTEIN (BJ6DN). Approximate amounts of antigen placed in each well are shown.

solutions prevented detection of slight turbidity. On heating in a boiling water bath flocculent precipitate appeared, in greater density in the serum solutions; filtrates from both solutions were clear and did not become turbid on cooling.

Agar precipitin patterns are shown in Figures 5 and 6. Unheated serum globulin solutions gave the heaviest precipitate in a narrow, straight zone. In addition, a second band, farther from the antiserum reservoir and convex to it, was usually discernible. This pattern remained unchanged after heating and filtering at 55° C. Filtrates obtained at 98° C never gave a visible precipitate.

Unheated urine globulins also gave a straight zone of precipitate. A second zone always occurred nearer the antiserum reservoir and concave to it; this consisted either of one broad band or of two parallel bands with little separation. Heating and filtering at 55° C regularly changed this pattern: the curved zones of precipitate became less dense and of less obvious curvature, and moved closer to or merged with the straight zone. With filtrates obtained at 98° C only the curved zone of precipitate appeared. This was always present, was similar to but fainter than the curved zone formed by unheated solutions, and gave with this zone a "reaction of identity" (20).

Precipitin-inhibition tests provided semiquan-

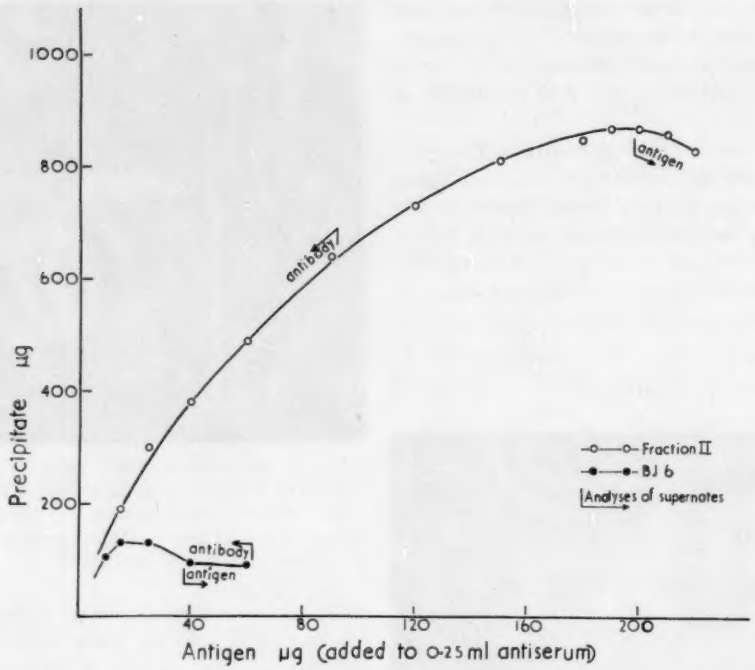

FIG. 3. QUANTITATIVE PRECIPITIN REACTIONS OF ANTI-II SERUM WITH FRACTION II AND A BENCE JONES PROTEIN.

titative confirmation of the above findings. Results using unheated solutions and 55° C filtrates are presented in Table IV. For each sample, the extents of precipitin-inhibition by equal volumes of unheated and heated solutions are compared. Serum globulins usually and urine globulins invariably showed decreased antigenic activity after heating. In the serum-urine pairs tabulated, urine components showed the greater decrease with significant frequency [$p = 0.03$ (21)]. Serum globulin filtrates obtained at 98° C never caused detectable precipitin-inhibition. Corresponding urine globulin filtrates (three from A, one from each other subject) gave detectable inhibition in all cases except that from F.

Some miscellaneous studies tested the possibility that observed differences between urine and serum gamma-related proteins could be due to dilution of gamma globulins or to urinary proteolytic action. Some sera (A1, A2, C) used in the above experiments were initially diluted 2,000-fold with buffered saline and then treated as detailed for urines. No differences were detected between these preparations and those in which this large dilution was omitted. Addition of 2 ml of serum or of 50 mg of Fraction II to 24-hour urine volumes, followed by incubation for six hours at 37° C, did not increase the amount of gamma-related protein subsequently found in 98° C filtrates of globulin concentrates. In each case there was a decrease, suggesting increased absorption by the greater amount of precipitate forming at 98° C.

Recoveries of urinary gamma-related protein soluble at 98° C were attempted after adding varying amounts of urine globulin solution to a serum globulin solution (globulins from 1 ml of serum in 2.5 ml of saline-acetate). At least a 12 hour output of urine globulin had to be added for subsequent 98° C filtrates to give a detectable precipitate with the antiserum in agar.

DISCUSSION

Filterings at 55° and 98° C were employed for their differential effects on concentrations of Bence Jones proteins and gamma globulins in

mixed solutions. Results of Experiment 1 give some indications of the likely utility of these procedures. Of further interest is the behavior of precipitates formed by heating Bence Jones proteins and washing at room temperature with a solvent in which the native proteins readily dissolve. These precipitates dissolve to a large extent at 98° C and, on cooling, leave in solution some protein which reprecipitates on heating at 55° C. This is consistent with the view that some

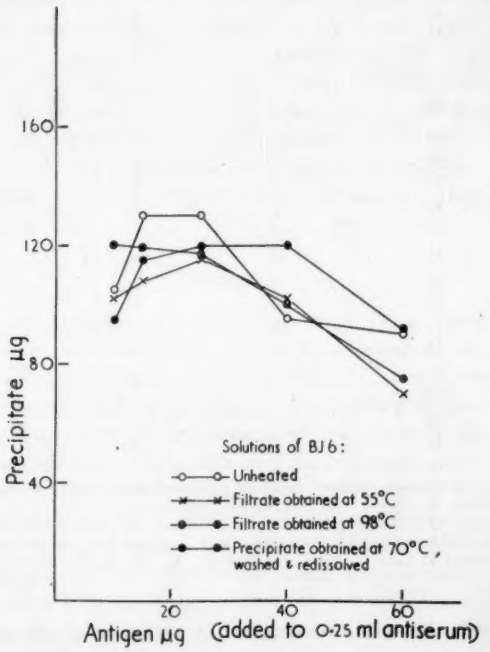

FIG. 4. QUANTITATIVE PRECIPITIN REACTIONS OF ANTI-II SERUM WITH VARIOUS PREPARATIONS OF A BENCE JONES PROTEIN.

dissolved denatured protein reverts to the native form on cooling from 98° C.

Bence Jones proteins heated at temperatures up to 98° C, if again obtained in solution at room temperature, were always antigenically indistinguishable from the native proteins. This is at variance with most immunological studies on denatured proteins [summarized by Boyd (22)] and implies either that change in antigenic structure on heat denaturation is minimal, or that any change is largely reversible. Ouchterlony plate appearances further suggest similar diffusion co-

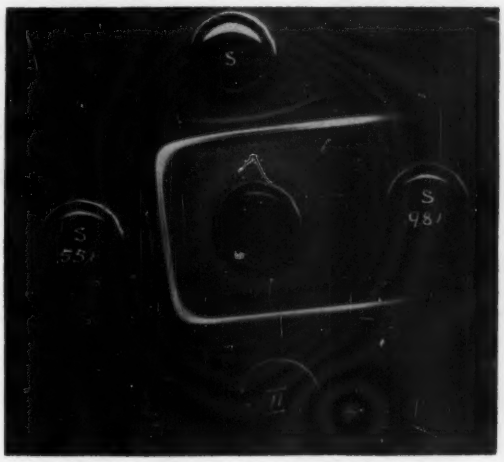

FIG. 5. AGAR PRECIPITATION PATTERNS OF ANTI-II SERUM WITH FRACTION II AND PREPARATIONS OF SERUM PROTEIN FROM SUBJECT B. S is unheated solution, S55F is the filtrate obtained at 55° C, and S98F is the filtrate obtained at 98° C.

efficients of native and heated products. The high solubilities of Bence Jones proteins at 98° C, together with persistence of original antigenic structure in the protein subsequently remaining in solution at room temperature, were the basis of the scheme originally devised for detection of a small amount of these proteins in the simultaneous

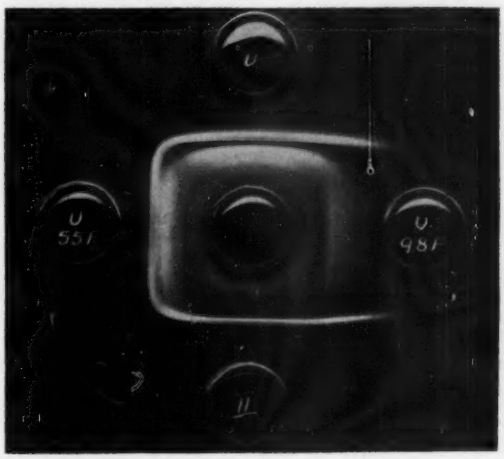

FIG. 6. AGAR PRECIPITATION PATTERNS OF ANTI-II SERUM WITH FRACTION II AND PREPARATIONS OF URINE PROTEIN FROM SUBJECT B. U is unheated solution, U55F is the filtrate obtained at 55° C, and U98F is the filtrate obtained at 98° C.

TABLE IV

Serum and urine globulins; unheated solutions and filtrates obtained at 55° C

Sample*	Precipitin-inhibition tests†			
	Antigen-excess endpoint for 0.2 ml supernate (equivalent to 0.1 ml antiserum) after incubation with		Change in endpoint on heating	
	Unheated solution	55° C filtrate	Tube difference	Turbidity on heating
	μg II	μg II		
Control (bovine serum albumin)	86			
A1 Serum	24	29	1	+
A1 Urine	24	35	2	+
A2 Urine [2]				−
A3 Serum [10]	35	35	0	+
A3 Urine [2]	42	>60	>2	+
A4 Urine [3]	42	60	2	−
A5 Serum	24	29	1	+
A5 Urine	24	35	2	+
B Serum	20	24	1	+
B Urine	20	29	2	+
C Urine [3]	24	35	2	−
D Serum	24	29	1	+
D Urine	24	35	2	+
E Serum [15]	24	24	0	−
E Urine [3]	29	35	1	−
F Serum	29	35	1	+
F Urine	29	35	1	−
G Serum	24	29	1	−
G Urine	29	42	2	+

* "Serum" refers to serum globulins dissolved in 5 ml of saline-acetate, "urine" to urine globulins dissolved in 1 ml. The solvent volume differed in some samples; for these, milliliters of solvent are indicated in brackets.

† Apart from Samples A3 and A4, the volumes of both unheated and heated solutions which were incubated with 1 ml of antiserum represented 0.1 total sample volume for urines, 0.02 total sample volume for sera. These were made up to 1 ml with phosphate-buffered saline. The amounts of Fraction II used for titration were 20, 24, 29, 35, 42, 50, 60, 72 and 86 μg.

presence of gamma globulin. However, the utility of the procedure is reduced by decreased recoveries of Bence Jones proteins in 98° C filtrates when other heat-precipitable protein is also present.

The results of Experiment 3 suggest the presence among urine globulins of protein resembling Bence Jones protein in *a*) reacting with antibodies to gamma globulin; *b*) having a higher diffusion coefficient, as evidenced by curvature of Ouchterlony precipitin lines, than has 7S gamma globulin; *c*) having a relatively high solubility at 98° C, with the solute, on cooling, antigenically resembling the native protein. To these similarities can be added the results of studies at 55° C, which are at least consistent with this protein's being readily denatured at this temperature. However, difficulties in detecting slight turbidity of urine solutions, and hence in correlating reduction in antigenic activity with this, and possible added complication due to the simultaneous presence of other proteins (23), indicate that interpretation of results from 55° C filtrates must be guarded.

Webb, Rose and Sehon (24) have reported physicochemical studies on electrophoretically separated urine gamma globulin, which suggest that this is virtually entirely represented by protein, antigenically related to serum gamma globulins, of molecular weight about 10,000. It is difficult to see how such a population could have given the Ouchterlony plate appearances reported here. It may represent breakdown products of larger molecules, or it may have been missed in the present work due to the fact that it was not pre-

cipitated by the ammonium sulfate (the concentration of which was chosen because it readily precipitates pathological Bence Jones proteins). These authors also give figures suggesting a daily gamma-related protein excretion of the order of 16 mg. This is much more than that suggested by the present study, in which protein reacting with the antiserum never exceeded 4.5 mg per 24 hour urine.

The origin of the Bence Jones-like protein is undetermined. No overt breakdown of serum gamma globulin by dilution or by urine to give such a product has been demonstrated. If it enters the urine in its defined form it could come from plasma or from some source in the urinary tract. The gamma-related protein described by Webb and associates (24) appeared, from isotopic studies, to originate from the serum gamma globulins.

SUMMARY

In attempts to detect micro-amounts of Bence Jones protein, or of protein resembling it, advantage was taken of reports that all Bence Jones proteins react with antisera to gamma globulins. Experiments were planned so that protein detected by reaction with such an antiserum could be further characterized by heat stability at 55° C, solubility at 98° C, and diffusion coefficients.

Two preliminary experiments provided information on the behavior of serum gamma globulins and Bence Jones proteins at 55° and 98° C. Evidence was obtained from macroscopic behavior on heating and from immunochemical studies of heated products, that heat denaturation of Bence Jones proteins may be largely reversible.

Evidence was found of the presence, in normal urine, of protein which resembles Bence Jones protein in reacting with antibodies to gamma globulin, having a similar diffusion coefficient and having a relatively high solubility at 98° C. Studies after heating at 55° C, although inconclusive, were consistent with a further resemblance to Bence Jones protein in that the protein was readily denatured at this temperature.

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ADDENDUM

Franklin (25) has provided further physicochemical studies of urinary protein of gamma electrophoretic mobility. He finds this to consist predominantly of a fraction which has a molecular weight of about 35,000 and is antigenically closely related to the serum gamma globulins; it was not thought to otherwise resemble Bence Jones protein. The suggested size of this protein is in accord with the present work, but some figures given by Franklin for its rate of excretion (his Table II) are much higher.

REFERENCES

1. Bence Jones, H. On a new substance occurring in the urine of a patient with mollities ossium. Phil. Trans. 1848, **138**, 55.
2. Jacobson, B. M., and Milner, L. R. Detection of urinary Bence Jones protein. Amer. J. clin. Path. 1944, **14**, 138.
3. Putnam, F. W. Aberrations of protein metabolism in multiple myeloma. Physiol. Rev. 1957, **37**, 512.
4. Rundles, R. W., Cooper, G. R., and Willett, R. W. Multiple myeloma. IV. Abnormal serum components and Bence Jones protein. J. clin. Invest. 1951, **30**, 1125.
5. Putnam, F. W., and Miyake, A. Proteins in multiple myeloma. VII. The free amino groups of Bence-Jones proteins. J. biol. Chem. 1957, **227**, 1083.
6. Deutsch, H. F., Kratochvil, C. H., and Reif, A. E. Immunochemical relation of Bence-Jones proteins to normal serum proteins. J. biol. Chem. 1955, **216**, 103.
7. Korngold, L., and Lipari, R. Multiple-myeloma proteins; III. Antigenic relationship of Bence Jones proteins to normal gamma-globulin and multiple-myeloma serum proteins. Cancer 1956, **9**, 262.
8. Thije, O. J. T. Urinary and serum proteins in myelomatosis. Acta med. scand. 1956, **153**, 253.
9. Snapper, I., Turner, L. B., and Moscovitz, H. L. Multiple Myeloma. New York, Grune and Stratton, 1953, p. 58.
10. McFarlane, A. S., Dovey, A., Slack, H. G. B., and Papastamatis, S. C. Unusual case of hyperglobulinaemia. J. Path. Bact. 1952, **64**, 335.
11. Hughes, J. T. Carcinoma of bronchus with Bence Jones proteinuria. Brit. med. J. 1954, **2**, 1267.
12. Collier, F. C., and Jackson, P. Precipitin test for Bence-Jones protein. New Engl. J. Med. 1953, **248**, 409.
13. Burtin, P., Hartmann, L., Fauvert, R., and Graber, P. Étude critique des techniques d'identification de la protéine de Bence-Jones et de leur valeur diagnostique. Rev. franç. Ét. clin. biol. 1956, **1**, 17.
14. Oncley, J. L. Physical characteristics of the gamma

globulins *in* Blood Cells and Plasma Proteins, James L. Tullis, Ed. New York, Academic Press Inc., 1953, p. 180.

15. Franklin, E. C., and Kunkel, H. G. Immunologic differences between 19S and 7S components of normal human gamma globulin. J. Immunol. 1957, **78**, 11.
16. Grant, G. H. The proteins of normal urine. J. clin. Path. 1957, **10**, 360.
17. Goa, J. Micro-biuret method for protein determination. Scand. J. clin. Lab. Invest. 1953, **5**, 218.
18. Kabat, E. A., and Mayer, M. M. Experimental Immunochemistry. Springfield, Ill., Chas. C Thomas, 1948.
19. Grabar, P., and Williams, C. A. Méthode permettant l'étude conjuguée des propriétés électrophorétiques et immunochimiques d'un mélange de protéines. Biochim. biophys. Acta 1953, **10**, 193.
20. Ouchterlony, Ö. Diffusion-in-gel methods for immunological analysis. Progr. Allergy 1958, **5**, 1.
21. Fisher, R. A. Statistical Methods for Research Workers, 13th ed. Edinburgh, Oliver and Boyd, 1958.
22. Boyd, W. C. Fundamentals of Immunology, 3rd ed. New York, Interscience Publishers, 1956, p. 118.
23. Kleczkowski, A. The formation of protein complexes in heated solutions of rabbit serum proteins. Brit. J. exp. Path. 1941, **22**, 188.
24. Webb, T., Rose, B., and Sehon, A. H. Biocolloids in normal human urine. II. Physicochemical and immunochemical characteristics. Canad. J. Biochem. 1958, **36**, 1167.
25. Franklin, E. C. Physicochemical and immunologic studies of gamma globulins of normal human urine. J. clin. Invest. 1959, **38**, 2159.

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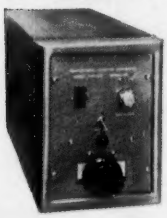

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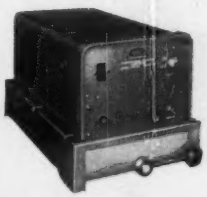

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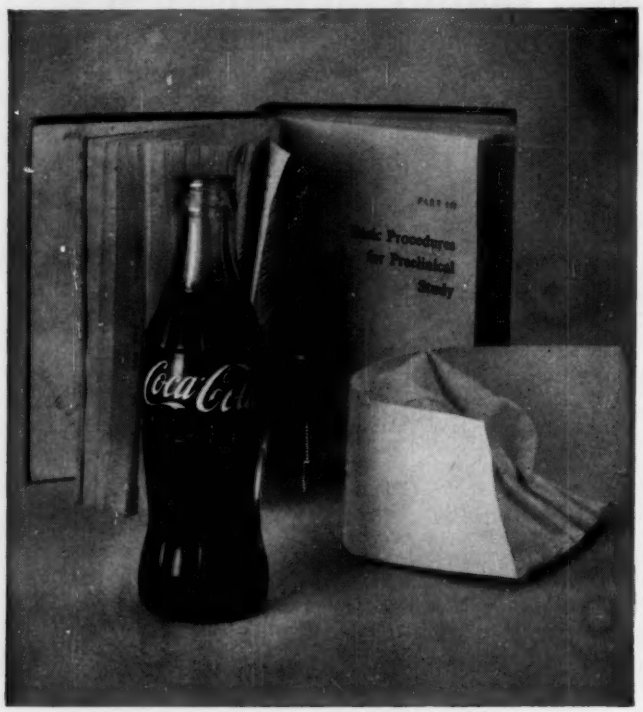

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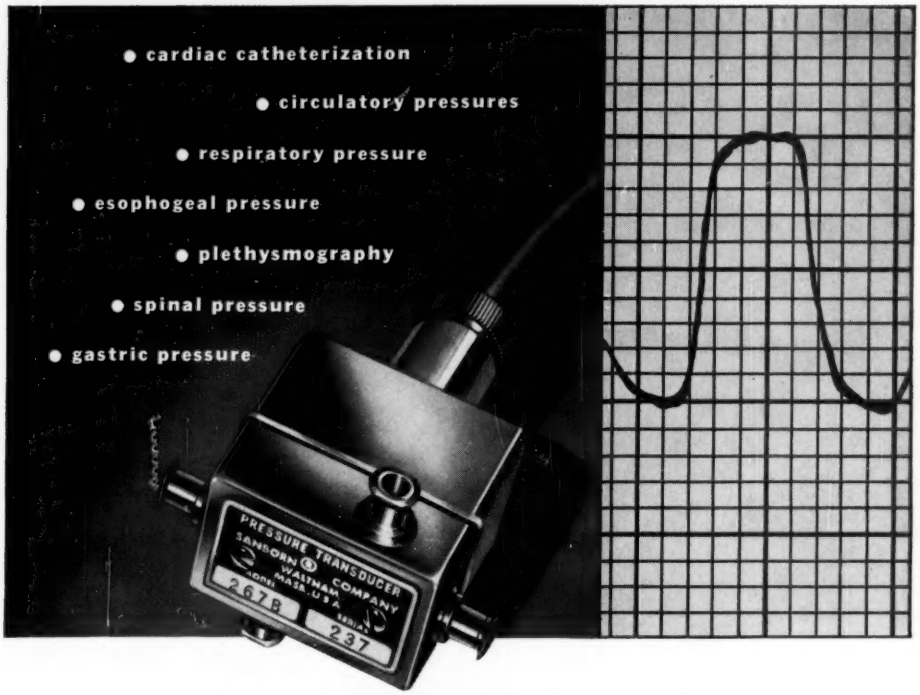

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1. Deutsch, E., and Christian, H.J.: J.A.M.A. *169*: 2012 (April 25) 1959.

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